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# HORMONAL MODULATION OF PHEROMONE-MEDIATED BEHAVIOR IN A CRUSTACEAN

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*Philadelphia, Pennsylvania 19104*

## ABSTRACT

A stereotyped courtship display is normally triggered in the male blue crab, *Callinectes sapidus,* by a pheromone released from pubertal females. Following bilateral eyestalk ligation, ablation, or optic tract transection, males do not respond to the pheromone, suggesting that neural pathways in the eyestalk ganglia are important for processing or transmitting pheromone stimulus information. Interestingly, males begin to exhibit spontaneous display behavior within a few days following eyestalk ligation or ablation, but not if only the optic tracts are transected. We propose that the loss of a circulating eyestalk factor, which moderates the activity of CNS pathways controlling courtship display, is responsible for the induction of the spontaneous behavior. This factor may normally control pheromone receptivity in males by modulating the excitability of these CNS pathways either directly or by acting via an intermediate(s); possibly by regulating the activity of the androgenic glands which exhibit massive hypertrophy following eyestalk ligation.

## INTRODUCTION

A fundamental goal of neuroethology is to understand the mechanisms which regulate the "behavioral state" of an animal (Truman and Weeks, 1985). Such regulation assures that the organism's behavior represents an appropriate interface between its internal physiological condition and the external environment. Hormonal controls are frequently of major importance in this regulation, particularly in reproductive behavior; although depending on the animal's reproductive strategy, social and environmental cues also can be of paramount importance (Crews and Moore, 1986). Pheromone communication plays a critical role in the reproductive activities of animals in nearly all major phyla (Shorey, 1976). Frequently these chemical messages trigger very specific stereotyped behaviors, which ultimately insure that mating is successful. In this report we consider a mechanism by which internal factors may regulate sensory activation of such a behavioral program.

The reproductive behavior of *C. sapidus* is coordinated in part by a pheromone that is present in the urine of pubertal females and detected by males via chemoreceptor sensilla (aesthetascs) on the outer flagellum of the first antennae (Gleeson, 1980, 1982). In receptive males this pheromone triggers a stereotyped courtship display which is characterized by lateral spreading of the chelae, extension of the walking legs, and lateral waving of the swimming appendages above the carapace. Immediately following the display, the male grasps the female and carries her beneath him for up to several days until she undergoes her maturity molt. Copulation is initiated soon

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thereafter, and this is normally the only time during her entire life that the female will mate.

In experiments which have focused on determining the chemical nature of this pheromone, males are routinely screened for pheromone receptivity before use in bioassays (Gleeson *et al.,* 1984). Interestingly, this screening procedure has revealed apparent cycles of receptivity within the male population; *i.e.,* at times very few males will display when presented with a pheromone stimulus, whereas at other periods nearly all males are responsive. No obvious correlations between these peaks of receptivity and seasonal mating activity, molt stage, or cycles of environmental parameters have yet been identified.

This apparent periodicity in receptivity suggested that hormonal controls may be operating. If so, the androgenic glands might be potential sources of such a regulatory factor since they are known to directly control the development and maintenance of both primary and secondary sexual characteristics in male crustaceans (Charniaux-Cotton and Payen, 1985). Because an eyestalk hormone (possibly gonad inhibiting hormone) has been shown to moderate the activity of the androgenic glands in several crustacean species (Adiyodi and Adiyodi, 1970; Adiyodi, 1985), we reasoned that ligating the eyestalks in male blue crabs may be an approach to increasing androgenic hormone production and consequently enhancing the males' pheromone receptivity; *i.e.,* if, in fact, an androgenic hormone affects receptivity. This hypothesis is only testable, however, if the olfactory pathways in the eyestalk ganglia [*e.g.,* the medulla terminalis (Ache and Fuzessery, 1979)] are not critical for processing pheromone stimulus information.

In this study we present evidence suggesting the existence of hormonal modulation of pheromone-triggered courtship display behavior in *C. sapidus* and begin to define the neural substrate affected by this modulation.

## MATERIALS AND METHODS

All animals were obtained from commercial sources, maintained in holding tanks with flow-through seawater systems, and sustained on a mixed diet of fish, squid, and shrimp which was offered 2–3 times weekly. Molt stages were assessed according to the criteria of Van Engel (1958).

Eyestalk ligations were performed by tightly tying off each eyestalk at its base in the region of the arthrodial membrane using a suitable length of 000 suturing silk. In animals in which the eyestalks were ablated, amputations were made just distal to a ligature tied at the base of each eyestalk; this procedure considerably reduced the loss of hemolymph from the stump. The ligation and ablation procedures are equivalent in their effects in that with ligation there is an immediate loss of visual input as judged by the animal's lack of response to visual cues that would normally trigger a defense posture. Furthermore, necrosis of eyestalk tissue is quite apparent within 24 h post-ligation with the eyestalk frequently becoming detached after a few days. Transections of the optic tracts were achieved using small scissors (Mini-Vannas) which were inserted through a transverse slit, approximately 2 mm in length, made in the mesial surface of the arthrodial membrane at the base of each eyestalk.

For behavioral observations, all animals were held individually in 40-liter aquaria, each having a flow-through seawater supply. Behavioral activity was monitored in one of two ways. In the first, observations were made daily for three 15-min periods (morning, midday, and afternoon) throughout the duration of the experiment. In a second approach, crab activity was automatically recorded on video cassette for 40 s intervals, every half hour, 24 h a day. Using this latter method, up to eight animals could be monitored simultaneously.

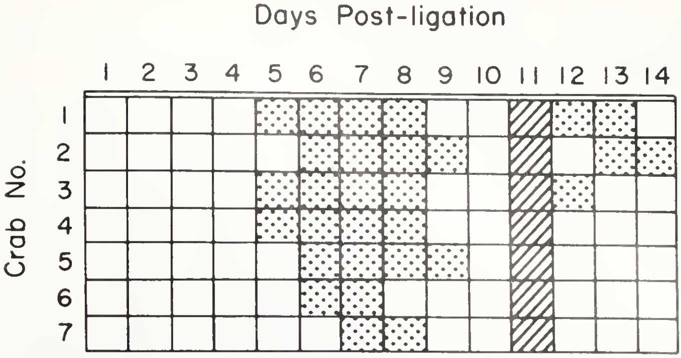

FIGURE 1. Occurrence of "spontaneous display behavior" (stippled squares) following bilateral eyestalk ligation of adult *Callinectes sapidus* males. All animals were observed for three 15 min periods daily. No data were collected on day 11.

Pheromone receptivity of males was tested by introducing a 300 μl sample of pubertal-female urine into the inlet flow of each aquarium and observing the male's behavior for two minutes. In all tests the urine samples were derived from pooled batches which, in our bioassay system, reliably trigger display behavior in intact, receptive males within two minutes.

For histological processing of the androgenic glands, the subterminal regions of the deferent ducts were dissected out, fixed with 2.5% glutaraldehyde in isosmotic *Callinectes* saline (Perkins and Wright, 1969) and refrigerated overnight. This was followed by treatment in Carnoy's fluid for 90 min at 4°C then dehydration and imbedding in paraffin. Tissue cross-sections (10 μm thick) were subsequently subjected to standard hematoxylin and eosin staining.

## RESULTS AND DISCUSSION

A pilot study to examine the effects of eyestalk ligation on pheromone receptivity was performed using four non-receptive adult males. In these trials a pheromone stimulus was presented to each animal, daily, for several days following bilateral eyestalk ligation. The results were surprising. Exposure to the pheromone during the first four days post-ligation did not trigger courtship behavior; however, on days five and six, three of the males began to exhibit courtship display activity *in the absence of a pheromone stimulus.* This behavior was, in all respects, identical to the stereotyped display activity that is normally triggered by the pheromone of pubertal females. Carrying behavior, which normally follows courtship display, was also initiated by these spontaneously active animals when other males were placed in their tanks. Bouts of the "spontaneous display behavior" (SDB), lasting up to several hours, occurred for approximately three days; thereafter the activity appeared to decline. Because of this spontaneous behavior, the results of tests to evaluate pheromone receptivity in these animals were inconclusive.

To explore this phenomenon further, an experiment was conducted using 30 intermolt males of mature size (carapace short-width = 113.3 mm, SEM ± 0.8), 15 of which were randomly selected for bilateral eyestalk ligations; the remaining 15 served as controls. All animals were observed three times daily over a 14 day period to monitor the occurrence of SDB. Of the 10 males surviving ligation, 7 exhibited SDB, whereas no such activity was observed in the 15 control animals (Fig. 1). The sponta-

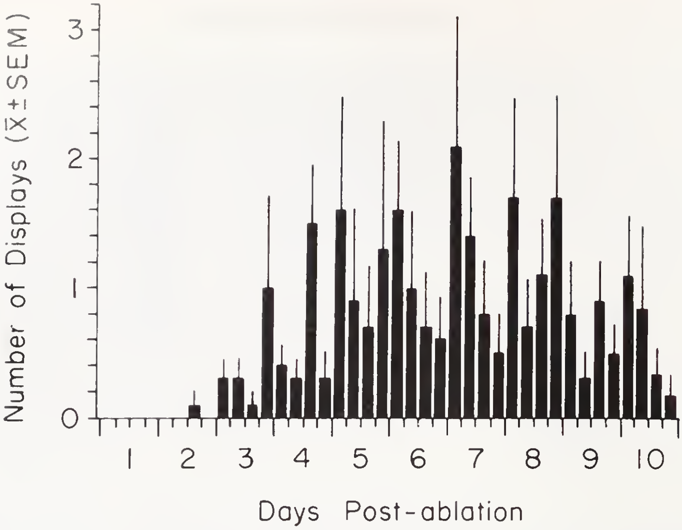

FIGURE 2. Frequency profile of "spontaneous display behavior" for 12 adult *Callinectes sapidus* males following bilateral eyestalk ablation. Behavior was sampled for 40 s intervals every half hour over 10 days. Shown are the mean number of intervals in which display activity occurred during each six hours post-ablation.

neous behavior was not seen until the fifth day after ligation, reaching a peak on day seven before subsiding.

The profile of this behavior was more precisely documented in a group of 12 adult, intermolt males (carapace short-width = 120.4 mm, SEM ± 1.2) in which the eyestalks were ablated. In this experiment, activity was automatically recorded on video cassette. The mean number of 40 s intervals in which SDB was observed during each 6 hours commencing on the day following ablation are shown in Figure 2. Again the profile reveals a delayed onset of the SDB with a maximum frequency between day five through eight, followed by a decline.

These results suggested that the eyestalks are sites for the production and/or release of a hormonal factor that modulates the activity of CNS pathways controlling courtship display behavior; *e.g.*, by regulating central pattern-generator circuits that coordinate the display. However, since the ligation/ablation procedure also removes a substantial portion of the crab's nervous system (*i.e.*, ganglia located in the eyestalks), an alternative hypothesis is that the procedure eliminates "inhibitory neural inputs" from these ganglia to courtship display "centers" located elsewhere in the CNS.

To distinguish between these alternatives, the optic tracts, which link the eyestalk ganglia with the brain (supraesophageal ganglion), were bilaterally transected in a group of 19 adult, intermolt males (carapace short-width = 117.8 mm, SEM ± 2.5). A second group of five adult, intermolt males (carapace short-width = 118 mm, SEM ± 4.7) received eyestalk ligations. The behavior of all animals was monitored three times daily over a ten day period. Dissection revealed that the transections were complete in 17 of the 19 animals subjected to the procedure, and in virtually all cases, hemolymph circulation to the eyestalks was not compromised as judged by the lack of necrosis in the eyestalk tissues 10 days after the operation. Of the 17 "transected" animals, only a single crab exhibited SDB. In contrast, four of the five "ligated" males

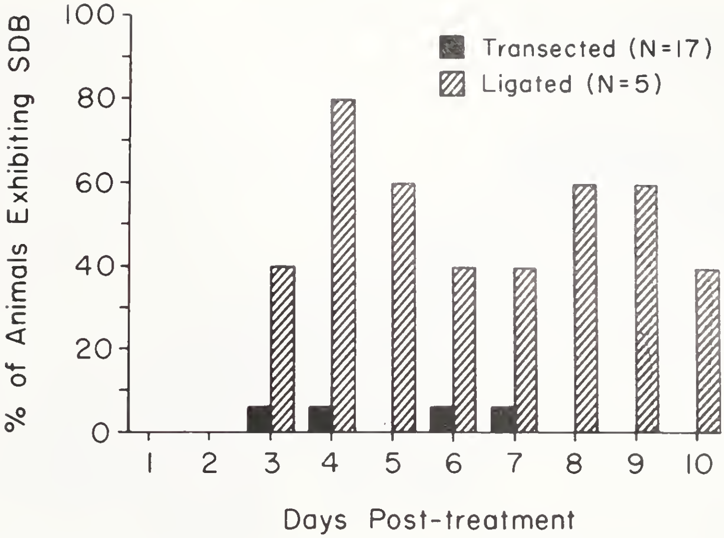

FIGURE 3. Occurrence of "spontaneous display behavior" (SDB) in adult *Callinectes sapidus* males following either bilateral eyestalk ligation (ligated) or optic tract transection (transected).

were induced to display (Fig. 3). The difference in the frequency of occurrence of SDB between experimental groups is significant ($P = 0.003$, Fisher exact test). These findings therefore demonstrate that the loss of any inhibitory neural inputs from the eyestalk ganglia is not a factor in the induction of SDB, and support the concept of an eyestalk hormone which acts to modulate this behavior. The latter view is a particularly tenable hypothesis considering that the transection procedure leaves only the integument and hemolymph linking the eyestalks to the animal. The operation therefore approximates (although it does not replace) the implantation of eyestalk tissue as a means of demonstrating the existence of a hemolymph-borne factor.

That the androgenic glands may be involved as possible intermediates in the induction of SDB is suggested by the hypertrophy they undergo following eyestalk ligation. The massive increase in size of the androgenic tissue depicted in Figure 4 is representative of what is consistently observed in adult, intermolt males subjected to the ligation procedure, and is quite apparent under the dissecting microscope as soon as day five following ligation. At least part of the increase in size can be attributed to hypertrophy of individual cells. Control animal tissue was characterized by cords of compact, linearly arranged cells; whereas in eyestalk-ligated males, the cytoplasm was typically more basophilic and greatly increased in volume. Under the light microscope, the appearance of the androgenic tissue in males actively exhibiting SDB was indistinguishable from that taken from animals up to 14 days post-ligation, a time at which the frequency of SDB activity has subsided. Hypertrophy was not observed in males in which the optic tracts were transected.

To assess the effects of eyestalk ligation on pheromone receptivity, 18 "ligated" males exhibiting SDB were screened for their response to pheromone-containing urine. The urine was presented during a period of quiescence between bouts of SDB. Only one of the eyestalk-ligated males, however, exhibited display behavior with urine presentation, and the response latency suggested that it may have been a bout of SDB. To explore this deficit in receptivity further, a group of seven highly receptive,

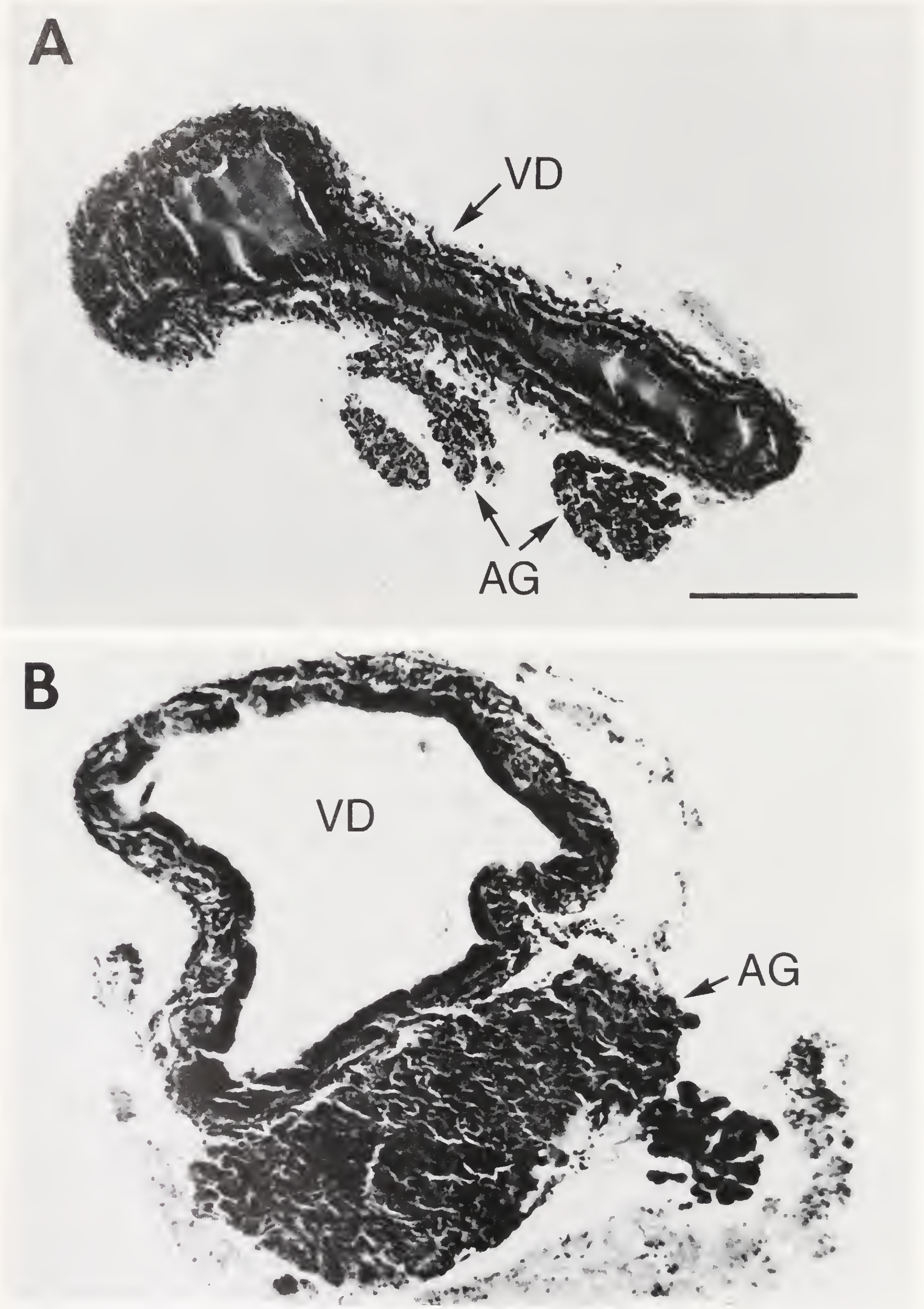

FIGURE 4. Cross-sections of the posterior vas deferens (VD) with associated androgenic gland (AG) from a control (A) and experimental male (B) 14 days following bilateral eyestalk ligation. Scale bar equals 200 microns.

intact males, which repeatedly exhibited vigorous display activity with urine presentations, were tested 24 h after either bilateral eyestalk ligation (two animals) or optic tract transection (five animals). In these trials, the pheromone stimulus did not induce any of the animals to display.

These results suggest, first, that disrupting the neural connections between the eyestalks and the supraesophageal ganglion blocks chemosensory activation of courtship display "centers" in the CNS; thus implying that neural pathways within the eyestalk ganglia are, indeed, important for processing or transmitting pheromone stimulus information. Two lines of reasoning support this view: (1) males which reliably respond to a pheromone stimulus immediately lose their receptivity if the neural connections to the eyestalks are severed; and (2) since the motor program for display behavior remains intact following eyestalk ligation/ablation as revealed by SDB activity, the lack of response to a pheromone stimulus in eyestalk-lesioned males cannot be attributed to the animal's inability to perform the display, implying that chemosensory activation of the program is in some way blocked. Interestingly, in several crustaceans bilateral eyestalk lesions have also been shown to profoundly attenuate food-search behavior elicited by chemical stimulation of the antennules (Maynard and Sallee, 1970; Hazlett, 1971). A second point to be made from these data is that the SDB observed in ligated males is not attributable to hyperexcitability of pheromone receptor cells in the antennules (although such hyperexcitability may in fact also occur), but rather involves changes in the excitability of CNS pathways mediating display behavior. This is further supported by the fact that in nine eyestalk-ligated males in which the antennules were also bilaterally ablated, SDB was nevertheless induced.

Considering the data together we propose the following relationships as a working hypothesis (Fig. 5). In receptive males, stimulation of antennular pheromone receptors normally activates CNS "centers" controlling courtship display; and in the pathway linking the antennular receptors with these "centers," neural connections within the eyestalk ganglia appear to be important components. We propose that the excitability of the display "centers," and consequently the threshold for activation by a pheromone stimulus, is modulated by an eyestalk hormone; possibly a neurosecretory product released into the hemolymph via the sinus gland. Exactly how this modulation is effected is not known. The action could be direct [*e.g.*, via the action of neuro-depressing hormone (Arechiga *et al.*, 1977, 1979)], and/or entail one or more intermediates. One possibility of the latter is that a hormone(s) from the androgenic glands is involved, as is suggested by the hypertrophy these glands undergo following eyestalk ligation. Indeed, a positive correlation between androgenic gland size and seasonal changes in the testes (Meusy, 1963; Payen, 1973), vas deferens (Adiyodi, 1985), and external male morphology (Carpenter and DeRoos, 1970; Thampy and John, 1973; Dudley and Jegla, 1978) has been noted in other crustaceans. Thus, if an androgenic hormone acts to turn on or increase the excitability of CNS courtship-display "centers," the eyestalk hormone may, in fact, be acting indirectly on these "centers" by modulating androgenic gland activity. Alternatively, the action of the eyestalk hormone may be mediated via other pathways; for example, by regulating the production or activity of a gonad stimulating hormone having CNS actions (Adiyodi and Adiyodi, 1970; Eastman-Reks and Fingerman, 1984; Kulkarni *et al.*, 1984).

The temporal profile of the SDB activity is intriguing and will quite likely provide an important clue towards understanding the mechanisms underlying this phenomenon. For example, the latency to onset of SDB may reflect the time required to appropriately raise the titer of a circulating androgenic hormone and/or reflect the time course of metabolic processes giving rise to the hyperexcitability in the neural elements affected by the hormone. The decline in SDB frequency by day 10 post-ligation

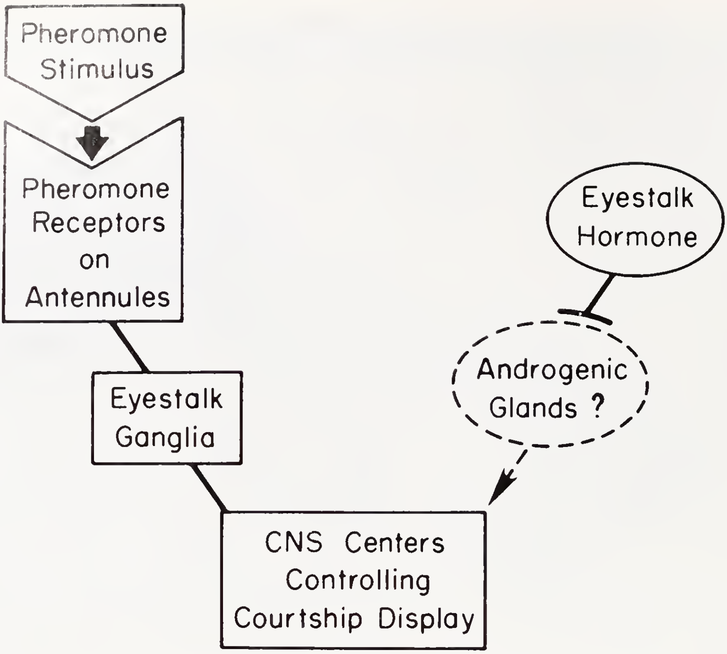
Pheromone Stimulus
Pheromone Receptors on Antennules
Eyestalk Ganglia
Eyestalk Hormone
Androgenic Glands ?
CNS Centers Controlling Courtship Display

FIGURE 5. Proposed model for activation and modulation of CNS "centers" controlling courtship display behavior. See text for discussion.

(Figs. 1, 2) may result from desensitizing mechanisms within the CNS or from compensatory metabolic processes which reduce the circulating titer of hormone. If the androgenic glands are indeed involved in the induction of SDB, the fact that their hypertrophy (and presumably hypersecretion) is evident well past the peak in SDB activity would suggest that mechanisms which compensate for a high titer of circulating androgenic factor are responsible for the reduction in SDB by day 10. Certainly, deciphering the exact mechanisms underlying SDB should provide important insight towards understanding processes regulating the sensory activation of behavior.

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# CRUSTACEAN CHEMICAL PERCEPTION: TOWARDS A THEORY ON OPTIMAL CHEMORECEPTION

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## ABSTRACT

Experiments explored how crustacea respond to odors that could signal energy and nutrient properties of food. While free amino acids are abundant in living prey, they diffuse rapidly from carrion and are assimilated by biodegradatory bacteria concomitant with release of ammonia. The ratio of amino acids to ammonia decreases with increasing carrion age and is proposed to signal the relative nutritional (nitrogen) quality of food. Supporting this hypothesis are data showing six bathypelagic and littoral species probing and searching to amino acids, but not to ammonia. Amino acids caused forward ambulation (searching) in some species while ammonia induced only tail-flipping (fleeing) in others. Interactions between amino acids and ammonia were clearly antagonistic, since mixtures combining these substances suppressed both feeding and fleeing. Additional experiments with the spiny lobster, *Panulirus interruptus,* demonstrate that excitation by the high energy molecule, ATP > ADP > AMP = adenosine, which is the pathway followed in autolytic degradation of animal flesh. Solutions identical in ATP evoked searching and feeding according to their ranked order of adenylate energy charge. Results indicate that crustacea respond best to odors that may specify food of highest energy and nutrient content, though responses differ between species and probably reflect differences in dietary habits.

## INTRODUCTION

A major problem for animals is how to exploit food resources efficiently. Ability to remotely sense food quality, quantity, and effort needed for food capture is clearly advantageous. This minimizes time choosing food and maximizes net rate of energy or nutrient gain (Hughes, 1979; Erichsen *et al.,* 1980; Orians, 1981). The decision to feed is based on many factors, including internal motivational states (level of starvation, social dominance, sex, and reproductive status; McFarland, 1971; Dethier, 1976; Harrison, 1983; Tilson and Hamilton, 1984; Bell *et al.,* 1985) and external environmental qualities, such as presence of predators or competitors (Milinski and Heller, 1978; Nelson and Vance, 1979; Barnard and Brown, 1981; Kacelnik *et al.,* 1981; Lima, 1985a; Schmitt and Holbrook, 1985). Foragers are influenced by stimuli specific to food, especially by smell and taste, which are vital to ingestion (Linstedt, 1971; Dethier, 1976; Ache, 1982; Croll, 1983, among others) and to activating and orienting search (Bell and Tobin, 1982). Chemical senses evolve to increase efficiency of resource use, given the goal of a forager is to minimize time spent acquiring set reward or is to maximize net rate of energy or nutrient gain.

Models of optimal foraging usually assume that grazers have complete prior knowledge of resource distributions, or that such knowledge is gained by cost-effective sampling. An implicit assumption is that foragers must distinguish the compara-

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tive worth of prey, usually by some unspecified mechanism in order to make the "right" energetic decision when grazing in a mixed prey assemblage. How sampling proceeds is poorly understood (but see Krebs *et al.,* 1978; Lima, 1985b), especially with regard to sensory mechanisms and perceptual ability.

This investigation determined how crustacea perceive chemicals that could specify energy and nutrient qualities of food. Marine crustacea were used because they depend almost exclusively on chemoreception in feeding (Hazlett, 1968; Schembri, 1982; Zimmer-Faust and Case, 1982a) and their behavior is highly stereotyped and easily quantified (Fuzessery and Childress, 1975; Field, 1977; Pearson *et al.,* 1979; Zimmer-Faust and Case, 1982b). Crustacea respond to a wide range of low molecular weight compounds that are readily synthesized (McLeese, 1970; Shelton and Mackie, 1971; Mackie, 1973; Carr, 1978; Robertson *et al.,* 1981; Zimmer-Faust *et al.,* 1984b), which makes them highly useful in chemical senses research.

### *A verbal model*

Free amino acids are abundant as osmolytes in tissues of all living aquatic invertebrates, the principal dietary items of crustacean omnivores (Clark, 1968; Gerard and Gilles, 1972; Clark and Zounes, 1977; Bowlus and Somero, 1979; Zurburg and DeZwaan, 1981; Yancey *et al.,* 1982). Because amino acids diffuse rapidly from carrion once a prey dies (Rittschof, 1980; Zimmer-Faust and Case, 1982a), they probably specify living or freshly killed prey. Free amino acids can be used as a source of nitrogen for protein synthesis and comprise as much as 8–10% of the dry weight of invertebrate flesh. Ammonia, on the other hand, is a nitrogenous waste product of transamination in protein catabolism and non-nutritive to crustacea (Campbell, 1973). Biodegradatory bacteria selectively assimilate other low molecular weight compounds, including amino acids (Ogura, 1975), while they produce copious amounts of ammonia (Hollibaugh, 1979; Kjosbakken *et al.,* 1983). The ratio of amino acids to ammonia decreases with increasing carrion age and decomposition (Kjosbakken *et al.,* 1983; Michel and Zimmer-Faust, unpubl. data); consequently, a corresponding reduction in predatory feeding might be expected. Interactions between amino acids and ammonia are proposed to signal the relative nutritional (nitrogen) quality of food.

Adenosine 5′-triphosphate (ATP) is involved either directly or indirectly with all cellular chemical energy transfers (Atkinson, 1977). Because ATP decays rapidly during cell death (Hiltz and Dyer, 1970), it is commonly used by ecologists to estimate "living" biomass (Holm-Hansen and Booth, 1966; Sinclair *et al.,* 1979; Ward and Cummins, 1979). Predator sensitivity to ATP alone cannot provide a reliable energy measure; cellular concentrations vary greatly depending on organismal life history stage, activity level, and on long-term seasonal factors (Bamstedt and Skjoldal, 1976; Sjkoldal and Bamstedt, 1976; Giesy and Dickson, 1981). Adenylate energy charge [AEC = (ATP + 0.5 ADP)/(ATP + ADP + AMP)] is a ratio of high energy phosphate bonds expressed as a function of the total phosphoadenylate pool. AEC scales from 0 to 1, with 1 indicating pure ATP (two high energy phosphate bonds) and 0 indicating pure AMP (zero high energy phosphate bonds). In normal healthy organisms (from bacteria to rats), an AEC of 0.8–0.9 is maintained even at the expense of compromising stored energy reserves. Ratios fall to ≤0.5 in dead, diseased, or in highly stressed organisms, only after energy reserves have been depleted (Chapman *et al.,* 1971; Balch, 1972; Ridge, 1972; Walker-Simmons and Atkinson, 1977; Ostolovskii, 1978; Buikema, *et al.* 1979; Ivanovici, 1980; Karl, 1980). Because AEC is indicative of the total metabolic energy available to an organism (Atkinson, 1977), its perception by a predator might assist in recognizing prey energetic quality. Predator sensitivity to ATP, as well as to AEC, was therefore expected.

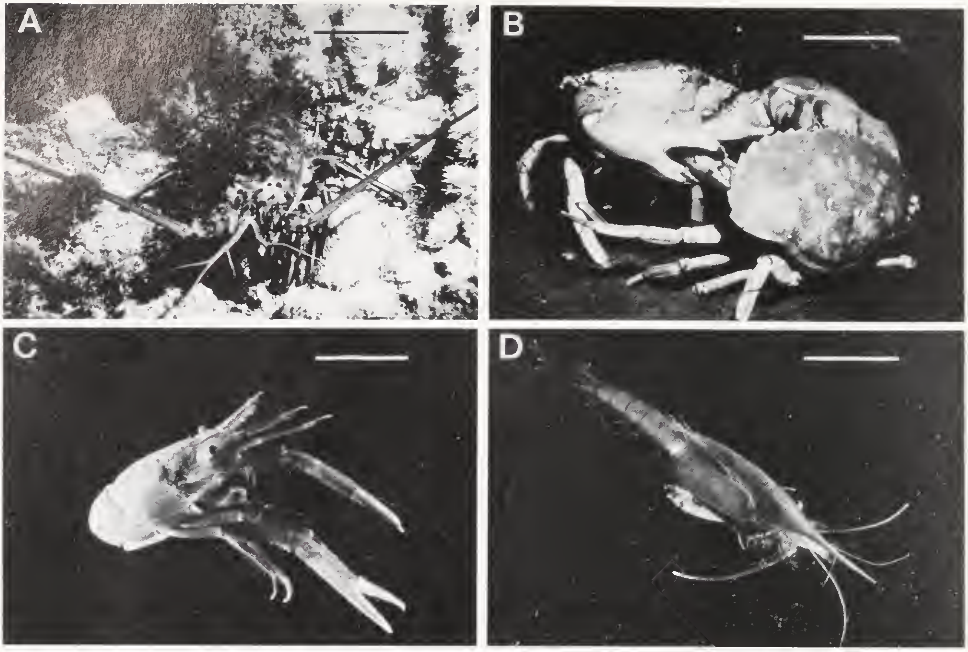

FIGURE 1. Animals representing four of six tested species, showing differences in morphologies and habitats. A. Spiny lobster, *Panulirus interruptus;* scale bar = 6 cm. B. Yellow crab, *Cancer anthonyi* (two individuals appear); scale bar = 6 cm. C. Pelagic red crab, *Pleuroncodes planipes;* scale bar = 2 cm. D. Deep sea mysid, *Gnathophausia ingens;* scale bar = 2 cm. Not shown are the hermit crab, *Pagurus hirsutiusculus,* and the majid crab, *Podochela hemiphilla.*

## MATERIALS AND METHODS

Species chosen for investigation presented a broad range of dietary preferences and habitats (Fig. 1 and Table I). Following collection, animals were held in 3000 l aquaria with flowing seawater at ambient temperature (15–17°C). A 12:12 D:L cycle (light on:0700 h) was imposed for two weeks. Animals were fed *ad libitum* on mackerel (*Scomber japonicus*) and salmon muscle (*Oncorhynchus* spp.), then food was withheld for 24 h prior to testing. Chemical solutions were presented to animals placed in plexiglas aquaria (30 × 30 × 15 cm), constructed to allow precise control of stimulus flow characteristics without inhibiting behavior (Zimmer-Faust and Case, 1983). A primary seawater flow (980 ml/min) entered each aquarium from a headtank maintained under constant hydrostatic pressure, while a secondary flow (120 ml/min) served to inject test substances (10 ml/7 s). Dilution associated with injection of a fluorescent dye was $1.02 \times 10^{-3}$ ($\pm 0.13 \times 10^{-3}$ SD) times the original concentration, as measured at the olfactory appendages (antennules) of test animals (see methods of Zimmer-Faust and Case, 1983). Presently reported concentrations are uncorrected for this dilution.

Test procedures were modified slightly for assays with *Gnathophausia,* since this animal required holding at an ambient temperature of 5°C. Individuals were maintained in 1-liter plastic containers and positioned in a constant temperature room (5°C), with seawater changed weekly. *G. ingens* has been maintained for up to 2.5 years under these conditions (Childress and Price, 1978). Test solutions (2 ml/10 s) were added directly to containers and seawater was changed following each trial.

TABLE I

*Collection histories of tested animals*

| Order<br>Infraorder<br>Genus species | Habitat | Depth (m) | Size (mm) ($\bar{x} \pm$ SD) | Principal dietary components |
|---|---|---|---|---|
| Decapoda | | | | |
| Palinura | | | | |
| *Panulirus interruptus* | Rock reef | 3–7 | $67.4 \pm 4.0^a$ | Animal flesh |
| Brachyura | | | | |
| *Cancer anthonyi* | Sand flat | 25–40 | $142.2 \pm 8.4^b$ | Animal flesh |
| *Podochela hemiphilla* | Pier piling | 2–5 | $13.8 \pm 2.0^a$ | Algae, animal flesh |
| Anomura | | | | |
| *Pagurus hirsutiusculus* | Rock intertidal | 0 | $5.8 \pm 0.8^a$ | Detritus, animal carrion |
| *Pleuroncodes planipes* | Midwater | 100–200 | $30.1 \pm 2.6^a$ | Animal flesh, zooplankton |
| Mysidacea | | | | |
| Lophogastrida | | | | |
| *Gnathophausia ingens* | Midwater | 400–600 | $32.4 \pm 3.4^a$ | Animal flesh, zooplankton |

[a] Measured as carapace length.
[b] Measured as carapace width.

### *Experimental procedures*

These were nearly identical to those previously described (Zimmer-Faust *et al.*, 1984b). Animals were tested a maximum of 4 times during a 14-day period, but only once each 72 h. They were put into experimental aquaria 30–60 min prior to testing and observed for 1 min before and for 4 min after chemical presentation. Trial length was chosen to maximize differences in responses to test and control stimuli, as determined in preliminary experiments. All tests were conducted using a double-blind protocol in which the observer was unaware of the composition of solutions introduced. Order of chemical presentation was established using a random numbers table with the exception that identical solutions were never repetitively introduced to the same animal. Solutions were prepared immediately before testing using analytical grade reagents and 0.45 $\mu$m filtered seawater, adjusted to pH 7.8.

Preliminary experiments evaluated responses to the amino acids, taurine, glycine, and glutamic acid. These were selected because of their proven ability to maximally excite crustacean feeding (McLeese, 1970; Fuzessery and Childress, 1975; Ache, 1982; Zimmer-Faust *et al.*, 1984b). Glycine was found most stimulatory to *Panulirus, Cancer, Pleuroncodes,* and *Gnathophausia,* while glutamic acid was most stimulatory to *Pagurus* and *Podochela.* These compounds were used as stimuli in the following tests.

### *Responses to amino acids and to ammonia*

Solutions of $10^{-2}$ *M* amino acid, $10^{-2}$ *M* ammonia, $10^{-2}$ *M* amino acid plus $10^{-2}$ *M* ammonia, and seawater, were presented to 24 specimens of each species. The injected concentration ($=10^{-2}$ *M*) produced a dilution-corrected $10^{-5}$ *M* in contact with the animals. This was appropriate given background levels of glycine and glutamic acid of $3 \times 10^{-8}$–$3 \times 10^{-7}$ *M*, and ammonia of $8 \times 10^{-7}$–$3 \times 10^{-6}$ *M*, in aquaria seawater with animals present (see Manahan *et al.*, 1983, for chemical analytical methods). Free amino acids are individually maintained at $10^{-4}$–$10^{-6}$ moles/g (dry weight), and ammonia at $10^{-6}$ moles/g, in the flesh of invertebrate prey consumed by crustacea (Clark and Hinkle, 1981; Zurburg and DeZwaan, 1981). Additional tests with *Panulirus* and *Pleuroncodes* investigated effects of ammonia concentration on amino acid responses. Solutions of $10^{-5}$, $10^{-4}$, $10^{-3}$, and $10^{-2}$ *M* ammonia were presented alone, and with $10^{-2}$ *M* glycine. Twenty animals from each species were tested with solutions. *Panulirus* was also tested with a mixture of 11 amino acids comprising >95% of the total free amino acid pool in abalone muscle, a preferred food (Zimmer-Faust *et al.*, 1984a). This was presented with, and without, $10^{-2}$ *M* ammonia added (n = 20 animals).

### *Responses to adenylates*

The spiny lobster, *Panulirus,* was further assayed for responses to ATP, ADP, AMP, and adenosine (ADO) at $10^{-4}$, $10^{-3}$, and $10^{-2}$ *M*. Tests were limited to lobsters because of time constraints and because adequate numbers of specimens were lacking for bathypelagic species. Twenty lobsters were tested with each solution. Subsequent trials employed a factorial design to present ATP, ADP, and AMP in all combinations. Each substance was maintained at $10^{-2}$ *M*. It was determined whether lobsters are best stimulated by the mixture of highest adenylate energy charge; by the mixture of highest total phosphoadenylate concentration; or by ATP (alone) independently of other phosphoadenylates. The injected concentration of $10^{-2}$ *M* produced a dilution-corrected $10^{-5}$ *M* in contact with animals. Individual phosphoadenylates are maintained at $10^{-4}$–$10^{-6}$ moles/g (dry weight) in the flesh of invertebrate prey consumed by crustacea (Hiltz and Dyer, 1970; Ansell, 1977).

### *Experiments with large tanks*

The above experiments poorly simulated natural conditions. This was especially true for *Panulirus* and *Cancer;* these animals were large relative to the size of test-aquaria. For this reason, several assays were repeated with isolated *Panulirus* and *Cancer* in large outdoor arenas (1.5 m diameter × 0.6 m water depth), with sandy substrates, shelters, and continuous flowing seawater (8 l/min). Experiments were performed at night under ambient moonlight (intensities = 0.02–0.06 $\mu W/cm^2$) with animals placed in arenas 24 h prior. Each animal was tested only once with a solution of $10^{-5}$ *M* glycine, $10^{-5}$ *M* ammonia, $10^{-5}$ *M* glycine plus $10^{-5}$ *M* ammonia, $10^{-5}$ *M* ATP, or seawater. These were pumped to the olfactory region (antennules) at 30 ml/180 s, through polyethylene tubing (0.32 cm ID) threaded in a hand-held acrylic rod. Sterile cotton was affixed at the tip to diffuse chemicals and to reduce mechanical disturbances caused by stimulus applications. Behavior was observed with an image intensifier and a 75 mm night vision lens (Javelin Electronics Model 221). A positive response was scored when an animal turned, walked towards, then attempted to grasp the cotton tip during a chemical presentation.

### *Addressing the question of mixture interaction*

Probability of response to a chemical presentation, $p(i + j)$, was a function of chemical [$p(i)$] and non-chemical [$p(j)$] factors (*e.g.*, spontaneous activity). Although $p(i)$ and $p(j)$ were assumed independent and binomially distributed, their distributions were not necessarily disjoint; consequently, $p(i)$ could not be directly measured. I used responses to seawater (control) in estimating $p(j)$ and determined $p(i)$, as follows:

$$p(i + j) = p(i \cup j) = p(i) + p(j) - p(i \cap j) \tag{1}$$

Because *i* and *j* were independent, $p(i \cap j) = p(i)p(j)$, and p(i) was solved algebraically:

$$p(i + j) = p(i) + p(j) - p(i)p(j)$$

and,

$$p(i + j) = p(j) + p(i)[1 - p(j)]$$

so,

$$p(i) = \frac{p(i + j) - p(j)}{1 - p(j)} \tag{2}$$

Binary mixture interaction between any two chemical agents, *a* and *b,* was evaluated by assuming that chemical effects were independent and additive. These assumptions were identical to those made by previous investigators who used "response addition" models to predict responses to mixtures (*sensu* Hyman and Frank, 1980). In this study the expected probability of response to a mixture, $p(i)_{a+b}$, was modelled by the equation,

$$p(i)_{a+b} = p(i)_a + p(i)_b - [p(i)_a p(i)_b] \tag{3}$$

An independent component index, f(R), was thus derived as:

$$f(R) = p(i)_{a+b} - \{p(i)_a + p(i)_b - [p(i)_a p(i)_b]\} \tag{4}$$

where $p(i)_{a+b}$, $p(i)_a$ and $p(i)_b$ were determined empirically. Independence between chemical effects was achieved at $f(R) = 0$, and $p(i)_{a+b} = p(i)_a + p(i)_b - [p(i)_a p(i)_b]$. Limits were established at $-1 \leq f(R) \leq 1$, given $0 \leq p(i) \leq 1$. Response synergy occurred from 0 to 1; suppression from 0 to −1; additivity occurred at zero. This index was similar to that of Hyman and Frank (1980), but with improvements made to account for the binomial nature of present data and to give equal weight to response synergy and suppression.

## RESULTS

Behavioral sequences defining early searching and appetitive phases of feeding are given in Table II, with descriptions taken from previous investigators wherever possible. For this study, it was necessary to derive behavioral sequences only for *Podochela* by watching animals before and after food presentations (= algal turf). I chose cheliped flexion (where applicable) or contact between antennules and mouthparts as criteria for "feeding," since these are exhibited stereotypically by crustacea (Mackie and Shelton, 1972; Snow, 1973; Fuzessery and Childress, 1975; Field, 1977; Pearson *et al.,* 1979; Zimmer-Faust and Case, 1982b; Trott and Robertson, 1984; Zimmer-Faust *et al.,* 1984a, b) and are of obvious utility either in contacting food, or in maintaining chemoreceptors functioning in orientation to food odor (Hazlett, 1968, 1971; Reeder and Ache, 1980; Devine and Atema, 1982). For *Gnathophausia,* I assayed alternating flexion and extension of endopodites on the maxillipeds as an appropriate indicator of feeding (Fuzessery and Childress, 1975). Locomotory behavior was monitored as movement of $\geq 1$ carapace length, either forwards or backwards.

### *Responses to amino acids and to ammonia*

Applications of amino acids, but not of ammonia, caused significant feeding responses relative to seawater (Fig. 2; G-Test for Independence with Williams' correction: $G \geq 10.49$, d.f. = 1, $P < 0.005$, comparisons for all six species). Independent component indices were all negative for mixture interactions (Fig. 3), and amino

TABLE II

*Sequential behavioral patterns in appetitive phases of feeding*

| Animal | Behavioral sequences |
|---|---|
| *Panulirus interruptus*[a] | (1) Antennule flicking rate is increased. (2) Pereiopods are extended and/or used to prod the substratum. (3) The third maxillipeds are flared and set into a lateral motion. (4*) Antennules are vertically deflected and brought into contact with the mouthparts. |
| *Cancer anthonyi*[b] | (1) Antennule flicking rate is increased. (2) Pereiopods are extended and/or used to prod the substratum. (3) The third maxillipeds are flared and set into a lateral motion. (4) Antennules are vertically deflected and brought into contact with the mouthparts. (5*) Chelipeds are extended and/or used to prod the substratum. |
| *Podochela hemiphilla*[c] | (1) Antennule flicking rate is increased. (2) The third maxillipeds are flared and set into a lateral motion. (3*) Chelipeds are extended and/or used to prod the substratum. |
| *Pagurus hirsutiusculus*[b] | (1) Antennule flicking rate is increased. (2) The third maxillipeds are flared and set into a lateral motion. (3) Pereiopods are extended and/or used to prod the substratum. (4*) Chelipeds are extended and/or used to prod the substratum, then brought to the mouth. |
| *Pleuroncodes planipes*[b] | (1) Antennule flicking rate is increased. (2) The third pair of maxillipeds are rubbed together. (3) The third pair of maxillipeds are outstretched and brought back to the mouth. (4) Pereiopods and chelipeds are extended and/or used to prod the substratum. (5*) Chelipeds are brought back to the mouth. |
| *Gnathophausia ingens*[b] | (1) Anterior endopodites are moved in a circular motion over the mouth. This motion appears to be related to filter feeding. (2) Endopodites are partially extended in an unconcerted manner. (3*) Endopodites are extended approximately perpendicular from the body in a concerted motion. (4) The abdomen is arched ventrally, either perpendicular to the body or with the telson almost in contact with the mouth. |

Sources of behavioral descriptions: [a]Zimmer-Faust *et al.*, 1984b; [b]Fuzessery and Childress, 1975; [c]present study.

Asterisks (*) denote defining acts.

acid stimulations of feeding were significantly suppressed by ammonia in *Panulirus, Cancer,* and *Podochela* [$p(i)_{a+b} < p(i)_a$, and G-Test for Independence with Williams' correction: $G \geq 4.29$, d.f. = 1, $P < 0.05$, all comparisons]. Similar results were obtained for *Panulirus* when the 11-component amino acid mixture was applied. This caused 70% of all tested animals to "feed" and 65% to walk, while only 35% "fed" and 10% walked following addition of ammonia (G-Test for Independence: $G \geq 4.92$, d.f. = 1, $P < 0.05$, both comparisons). The number of times a defining behavioral act was performed in each trial provided a measure of response intensity. For *Cancer, Pagurus,* and *Pleuroncodes,* response intensities evoked by amino acids were significantly depressed by ammonia (Table III; Student's two-tailed *t*-test for small sample sizes, variances assumed equal: $P \leq 0.08$, all comparisons). The intensities exhibited by *Panulirus* and *Podochela* were only slightly affected by ammonia ($P > 0.20$).

Littoral species walked forward to amino acids but not to ammonia, while bathypelagic species tail-flipped backward (fleeing) to ammonia but not to amino acids (Fig. 2). Mixtures reduced significantly the initiation of walking by *Panulirus,* and fleeing by *Pleuroncodes* and *Gnathophausia* [Fig. 3; $p(i)_{a+b} < p(i)_a$ and G-Test: $G \geq 4.14$, d.f. = 1, $P < 0.05$, for all comparisons]. The amino acids and ammonia acted antagonistically though which stimulated while the other suppressed depended on the behavior in question (walking or fleeing, respectively).

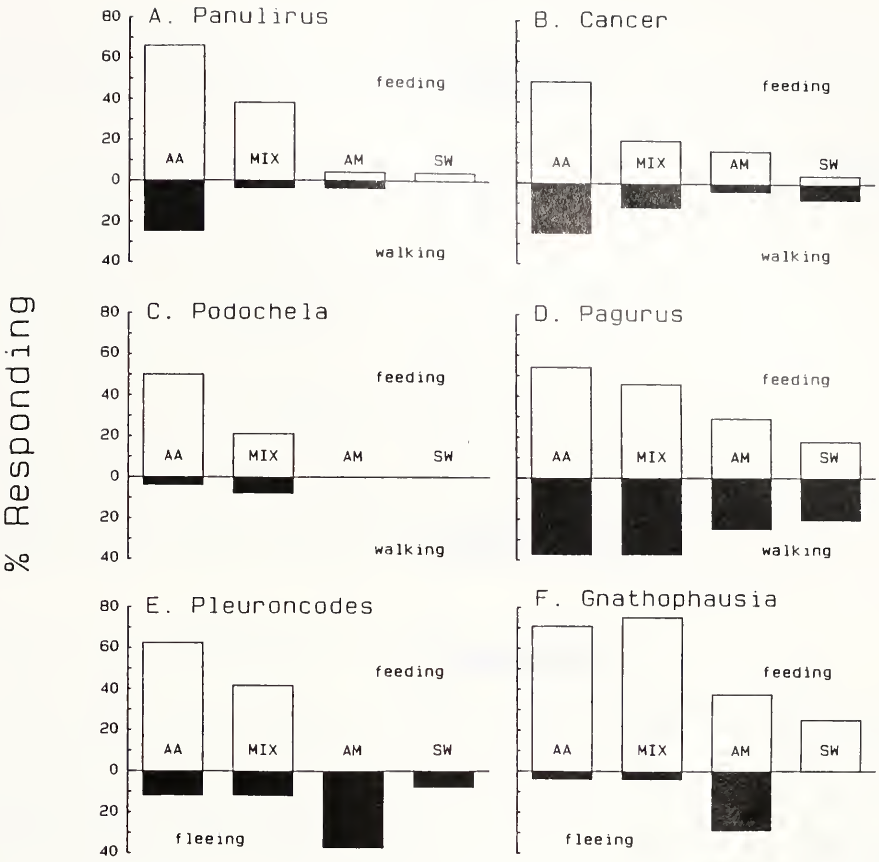

FIGURE 2. Feeding and locomotory responses to amino acid (AA), ammonia (AM), amino acid + ammonia (MIX), and seawater (SW). Each chemical was presented at $10^{-2}$ *M*. Forward ambulation (walking) was exhibited only by littoral, and tail-flipping (fleeing) only by bathypelagic species.

Ammonia was slightly inhibitory at $10^{-4}$ *M*, and significantly so at $10^{-3}$ *M* to glycine-induced feeding by *Panulirus* and chela probing by *Pleuroncodes* (Fig. 4; $P < 0.05$). Ammonia inhibited walking by *Panulirus*, just as it stimulated fleeing by *Pleuroncodes* at the same concentrations ($=10^{-3}$–$10^{-4}$ *M*) (Fig. 5). Dose-response data show ammonia consistently inhibitory or repellent over a broad range of concentrations.

## *Responses to adenylates*

Adenosine triphosphate was highly stimulatory to *Panulirus* at $10^{-2}$ *M* (G-Test: $G \geq 6.50$, d.f. = 1, $P < 0.01$, comparisons for feeding and walking), while ADP was only slightly stimulatory ($P > 0.10$). Both AMP and ADO were without stimulatory effects (Fig. 6). Stimulation by ATP was not independent of the other phosphoadenylates, since differences were nearly significant between the responses to ATP, ATP + ADP, ATP + AMP, and ATP + ADP + AMP (Fig. 7A; G-Test for Independence with Williams' correction: $G = 7.16$, d.f. = 3, $P = 0.06$, for feeding). It was proposed

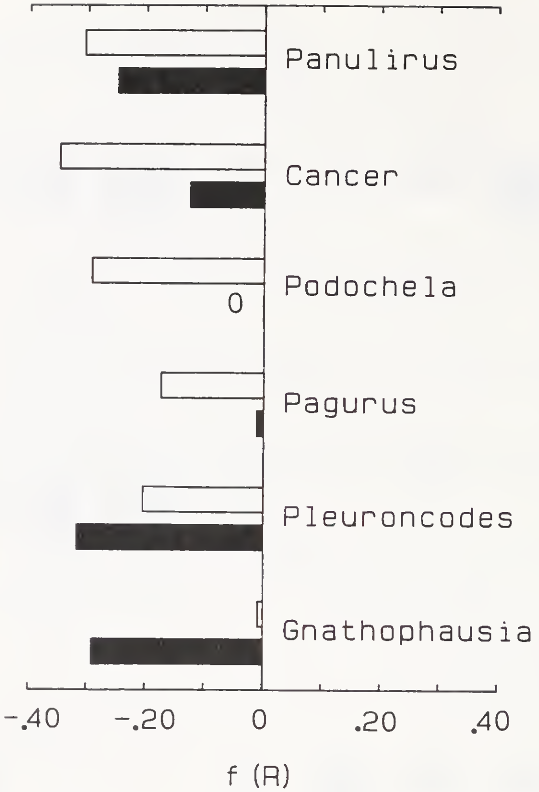

FIGURE 3. Independent component indices for interactions between amino acids and ammonia. Open bar (□), feeding; closed bar (■), locomotion (walking or fleeing). Negative values indicate suppression.

that ADP and AMP might enhance ATP stimulation according to the increase in adenylate concentration. This predicted that proportions of responding animals should be ranked, ATP + ADP + AMP > ATP + ADP = ATP + AMP > ATP. Observed rankings showed no positive association with that predicted (Kendall's one-tailed Tau, with correction for ties: $T \leq -0.40$, $n = 4$, $P > 0.80$, comparisons for feeding and walking; Accept $H_0$: no association). It was alternatively postulated that ADP and AMP might inhibit ATP stimulation according to the decrease in adenylate energy charge. This predicted that proportions of responding animals should be ranked, ATP > ATP + ADP > ATP + ADP + AMP = ATP + AMP = ADP > ADP + AMP > AMP. Because the observed rankings correlated positively only with those predicted by this last hypothesis, lobster chemosensitivity to adenylate energy charge is indicated (Kendall's one-tailed Tau, with correction for ties: $T \geq 0.827$, $n = 7$, $P < 0.02$, comparisons for feeding and walking; Reject $H_0$: no association, accept $H_a$: positive association).

Stimulation by ATP was not inhibited significantly by ADP (Fig. 7B; G-Test for Independence with Williams' correction: $G \leq 2.85$, d.f. = 1, $P > 0.10$, both comparisons), though it was by AMP [$p(i)_{a+b} < p(i)_a$, and G-Test: $G = 4.65$, d.f. = 1, $P < 0.05$ for feeding]. The stimulation by ADP was independent of AMP.

TABLE III

*Response intensities ($\bar{x}$ ± SEM) to applied chemical solutions*[a]

| Animal | Behavior | Chemical solution | | | |
|---|---|---|---|---|---|
| | | Amino acid | Amino acid + ammonia | Ammonia | Seawater (control) |
| *Panulirus interruptus* | Antennule wiping | 8.8 ± 2.0<br>(n = 17) | 11.4 ± 3.5<br>(n = 10) | — | — |
| *Cancer anthonyi* | Cheliped flexion | 4.4 ± 0.4<br>(n = 12) | 2.9 ± 0.3<br>(n = 5) | — | — |
| *Podochela hemiphilla* | Cheliped flexion | 8.4 ± 2.5<br>(n = 12) | 7.3 ± 3.4<br>(n = 5) | — | — |
| *Pagurus hirsutiusculus* | Cheliped flexion | 34.0 ± 5.5<br>(n = 13) | 23.0 ± 4.4<br>(n = 11) | 16.4 ± 7.4<br>(n = 7) | 13.0 ± 1.5<br>(n = 4) |
| *Pleuroncodes planipes* | Cheliped flexion | 6.0 ± 0.8<br>(n = 15) | 3.9 ± 0.5<br>(n = 10) | — | — |
| *Gnathophausia ingens* | Endopodite extension | NOT DETERMINED | | | |

[a] Data are the numbers of acts performed in 4 min trials. Values appear only where more than one of 24 tested animals responded. Parentheses denote the number of responding animals.

### *Experiments with large tanks*

*Cancer* was highly responsive to glycine but less so to the glycine-ammonia mixture (Fig. 8A). An independent component index of −0.240 for this interaction was nearly identical to those of −0.142 and −0.367 calculated from tests using smaller aquaria (Fig. 3). *Panulirus* was also highly responsive to glycine, as well as to ATP (Fig. 8B). The interaction between glycine and ammonia was again inhibitory and produced an index of −0.465, or nearly the same as before (−0.240 and −0.301, see Fig. 3). Experiments with large tanks confirmed the results from tests using smaller aquaria.

## DISCUSSION

Although chemosensory responses in this study were products of chemicals applied individually, significant mixture interaction occurred which caused behavioral suppression. Suppression is commonly reported in psychophysical investigations of odor and taste perception (Moskowitz, 1972; Berglund, 1974; Bartoshuk, 1975, 1977; Carefoot, 1982), though present findings go further in proposing an ecological function. Suppression gives control over the modulation and tuning of feeding according to overall perceived quality of a chemical signal. I propose that interactions between chemical agents function, in part, by tuning responses to relative energy and nutrient properties of odor. Mixture suppression might then benefit a searching organism by reducing its response to lower quality foods. Such behavior would be most advantageous under conditions where prey are relatively abundant and food is non-limiting, and where the optimal response is to graze selectively.

It might be argued that suppressant interactions between single amino acids and ammonia are relatively meaningless, given that prey flesh is naturally a complex chemical mixture. However, the single stimulants used in this study were those maximizing feeding and locomotory responses. By using binary mixtures in behavioral assays the complexity of ensuing electrophysiological analysis is greatly reduced when

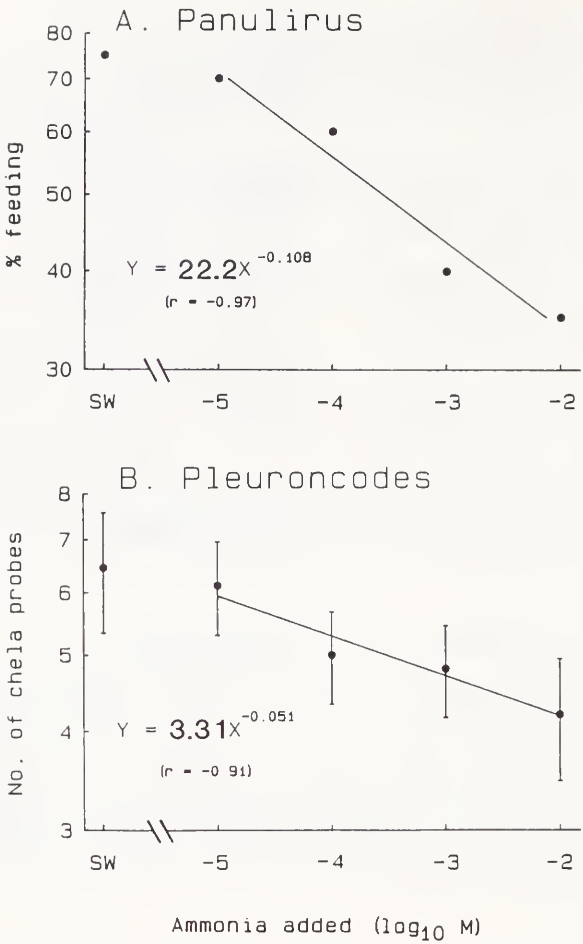

FIGURE 4. Effects of ammonia on $10^{-2}$ $M$ glycine-induced (A) feeding by *Panulirus*, and (B) cheliped flexion ($\bar{x} \pm$ SD) by *Pleuroncodes*. Responses are adequately modelled as power functions of ammonia concentration. No responses were observed to ammonia presented by itself at $10^{-5}$–$10^{-2}$ $M$. SW: No ammonia added.

considering the neurobiological sites of mixture interaction, *i.e.*, primary receptor or central nervous system. A mixture comprising >95% of the free amino acids in abalone muscle was highly stimulatory to *Panulirus*, but this activity was significantly reduced by the addition of ammonia. Previously, I found a low molecular weight fraction of abalone muscle (<1000 daltons) to be without stimulatory effect to lobsters (Zimmer-Faust *et al.*, 1984a). Experiments later demonstrated that this results from the presence of chemical suppressants, of which ammonia predominates, and not from a lack of stimulatory agents (Zimmer-Faust *et al.*, 1984b).

I interpret results from amino acid and ammonia assays in the following way. Amino acids alone probably trigger attack behavior and intensify within-patch search. Both claw and leg probing were evoked in response to amino acids, and these behaviors are essential to acquiring food in the near-field environment. The amino

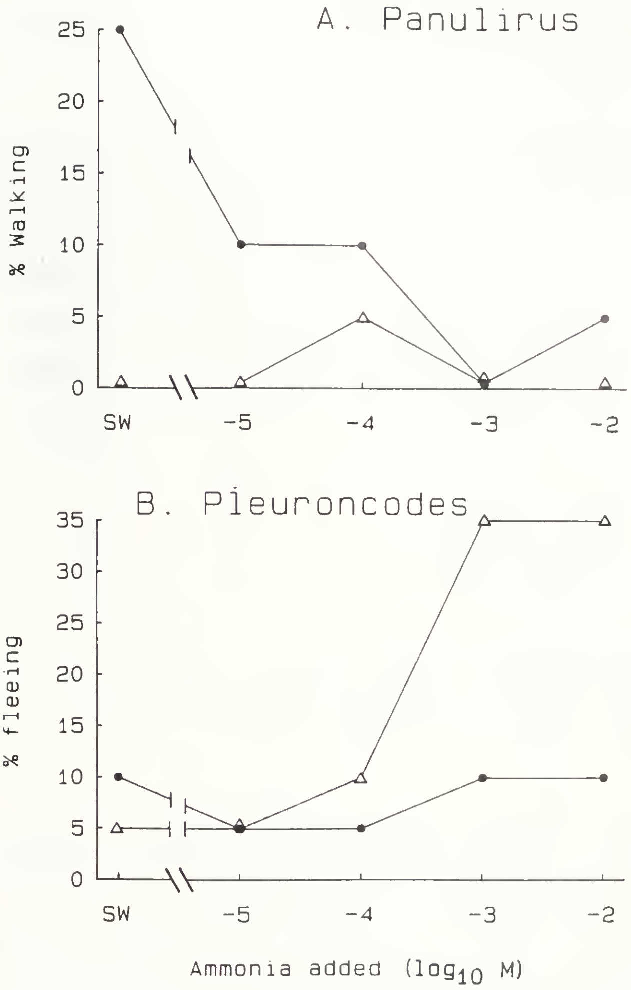

FIGURE 5. Effects of ammonia and glycine on locomotion. A. Walking by *Panulirus* is stimulated by glycine and suppressed by ammonia. B. Fleeing by *Pleuroncodes* is stimulated by ammonia and suppressed by glycine. Closed circles (●) are responses to ammonia with $10^{-2}$ *M* glycine added; open triangles (△) are responses to ammonia presented by itself. SW: No ammonia added.

acid induction of walking appeared to possess a klinokinetic or chemotactic component, since in large tank experiments, *Panulirus* and *Cancer* turned precisely towards a stimulus before moving to grasp the source. Ammonia alone probably causes patch departure. This was demonstrated for bathypelagic species; they tail-flipped away from ammonia. Small tank experiments suggest that littoral species may ignore ammonia, but these experiments did not test animals in an endogenously active state. Large tank experiments confirm that paths of actively moving lobsters (*Panulirus*) and crabs (*Cancer*) are unaltered by ammonia (Zimmer-Faust, unpubl. analyses of video data). Endogenous movements would carry these animals to alternative patches in natural habitats.

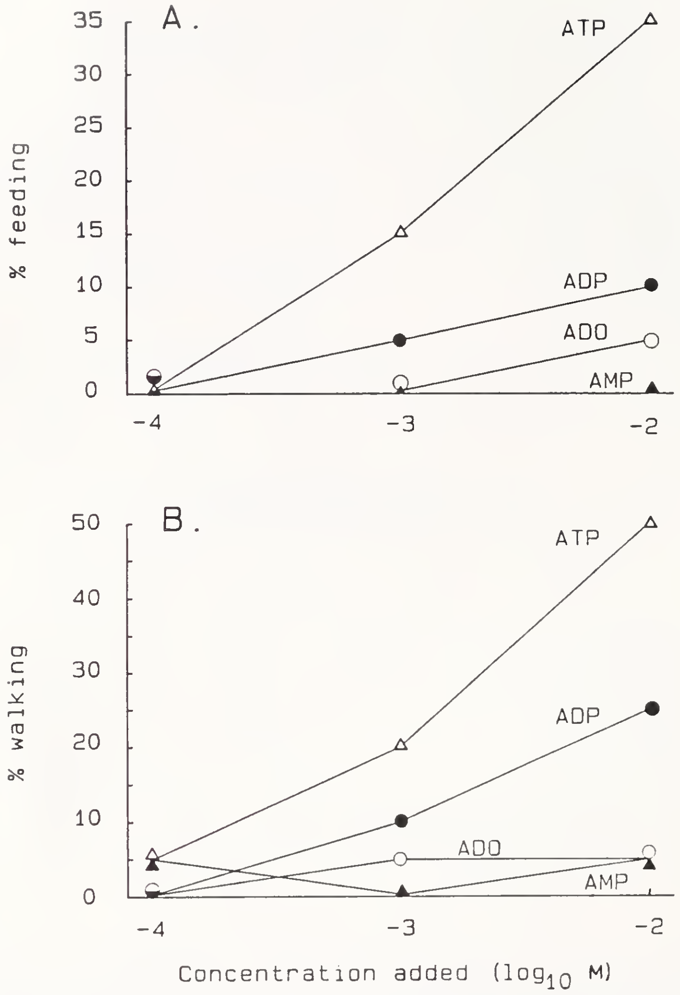

FIGURE 6. Dose-responses for (A) feeding, and (B) walking by *Panulirus* to the adenylates: ATP, ADP, AMP, and adenosine (ADO).

Mixtures suppressive to the initiation of feeding by *Panulirus* and *Podochela* were not inhibitory to the response intensity. The opposite was true for *Pagurus* since response intensity, but not initiation, was suppressed. Both *Panulirus* and *Podochela* typically move long distances while grazing. Their assessments of prey quality and density are likely to occur on the patch-level of resource organization, where a "stop and feed" rule is most advantageous. *Pagurus,* on the other hand, is a microphagous detritivore that is active yet limited in mobility due to its small size. Given the limitations in the patch-selection ability of *Pagurus,* variable ingestion rates may be the most important animal response affecting feeding energetics. An optimal foraging model predicts that the net time rate of energy gain is maximized when benthic deposit- and suspension-feeders covary ingestion rates positively with food value (Taghon, 1981). This is because such animals are unable to sample their environments and hence to choose among food items to the same degree that highly mobile predators

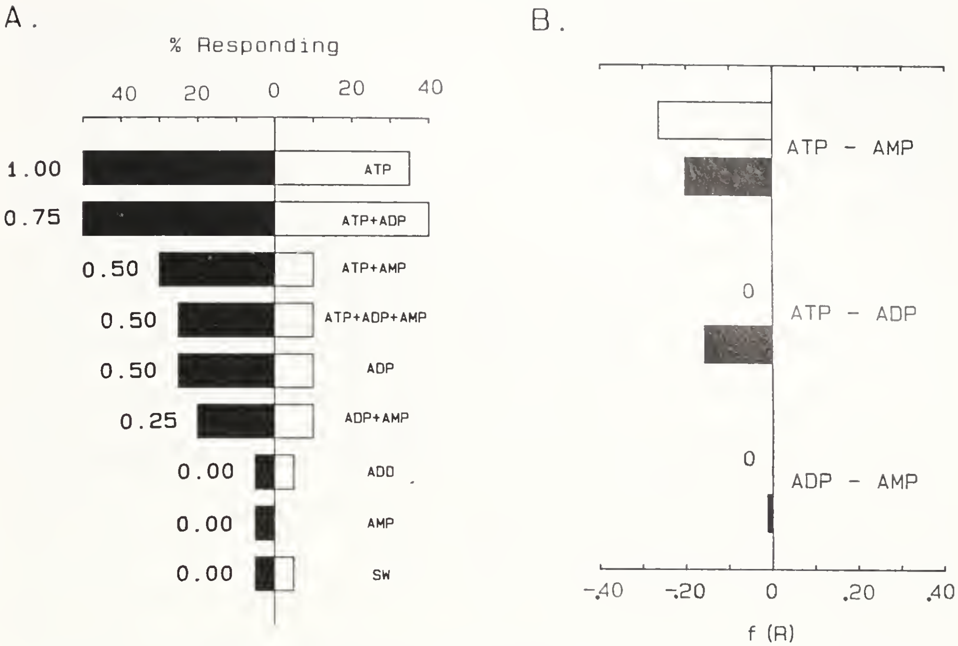

FIGURE 7. A. Feeding and locomotory responses by lobsters to single adenylates and mixtures. Values for adenylate energy charge appear (0–1 scale). B. Independent component indices for binary interactions between phosphoadenylates. Each chemical was presented at $10^{-2}$ *M*. Open bar (□), feeding; closed bar (■), walking.

can. Benthic marine organisms lacking mobility are dependent on food being brought to them by water currents, by lateral and vertical advections (Taghon *et al.*, 1980), and by microbial regeneration within sediments (Levinton and Lopez 1977; Newell, 1979).

By responding to ATP, *Panulirus* is similar to many of the haematophagous insects. Among Diptera, three species of culicine mosquitoes, the blackfly *Simulium venustum* (Smith and Friend, 1982), the tsetse fly *Glossina austeni* (Galun and Margalit, 1969), and the horsefly *Tabanus nigrovittatus* (Friend and Stoffolano, 1983) all require ATP or ADP as a phagostimulant. Responses by insects are thought to be adapted to the release of phosphoadenylates, particularly of ATP and ADP, associated with recruitment of blood platelets to open wounds. Apparent sensitivity by *Panulirus* to adenylate energy charge [AEC = (ATP + 0.5 ADP)/(ATP + ADP + AMP)] could enable it to assess the total metabolic energy available to a prey (Atkinson, 1977). Chemical excitation of lobsters was ATP > ADP > AMP = adenosine, which is the pathway followed by phosphoadenylates in autolytic degradation of animal flesh (Hiltz and Dyer, 1970). Dephosphorylation of ATP proceeds rapidly during cell death; also, stress related to disease, injury, and starvation elicit physiological defense responses (Selye, 1976) that increase metabolic activity and ATP use (Chapman *et al.*, 1971; Balch 1972; Walker-Simmons and Atkinson 1977; Ostolovskii 1978; Buikema *et al.*, 1979).

Optimal foraging theory usually predicts that grazers should become more selective at high food densities and less selective at low food densities (Pulliam, 1974; Pyke *et al.*, 1977; Owen-Smith and Novellie, 1982; Pyke, 1984). A corollary is that food

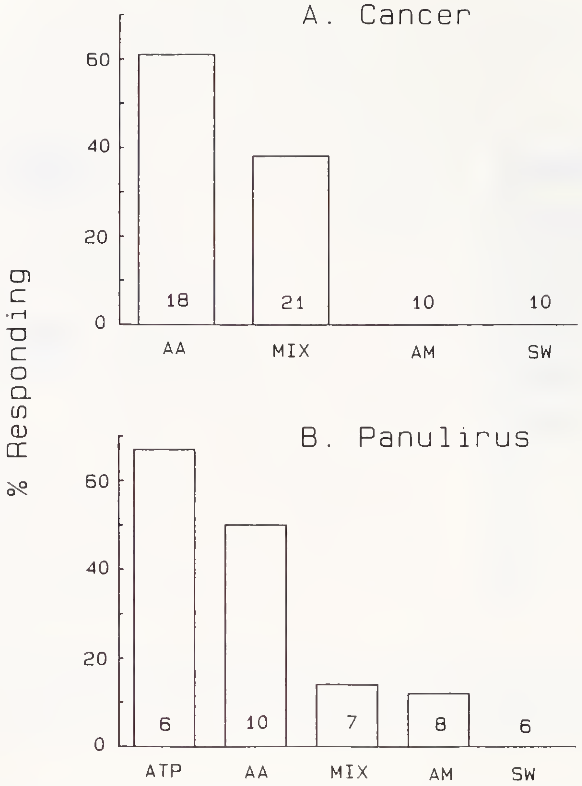

FIGURE 8. Responses to ATP, glycine (AA), ammonia (AM), glycine + ammonia (MIX), and seawater (SW), by (A) crabs and (B) lobsters. Animals were placed in large tanks, and each chemical was presented at $10^{-5}$ *M*. The number of animals tested is shown.

selectivity should decrease with increasing food deprivation and starvation, if recognition or assessment of food availability is modified by hunger levels (Snyderman, 1983). When grazers approach satiation they should behave as if food were abundant and become more selective (Schoener, 1971; Pulliam, 1974; Charnov, 1976). In this study, animals were maintained near satiety and they were without risk of death due to starvation. Therefore, chemical preferences might be expected for odors that signify food of highest energy and nutrient payoffs. Optimal foraging theory predicts that feeding responses should become more broadly tuned as food deprivation increases (or as prey densities are lowered), and as energy reserves are spent. That is, we should expect mixture suppression by ammonia and AMP to decrease as food becomes limiting. Mixtures should become more attractive at lower adenylate energy charges and at lower amino acid:ammonia ratios. These predictions have yet to be tested.

Caution is urged in developing hypotheses of "optimal" chemoreception. Many factors operate that could confuse experimental design and render test results meaningless. For example, optimality only can be tested fairly under the constraint that animals behave *given* their sensory, motor, and CNS capabilities. It is AMP rather than ATP that acts as a potent chemoattractant to the grass shrimp, *Palaemonetes pugio* (Carr and Thompson, 1983). Because this animal principally scavenges dead animal and detrital materials (Walsh, 1975), the AMP response may be adapted to the dietary mode and need not exclude an optimal chemoreception hypothesis. Differences in adenylate responses by *P. interruptus* and *P. pugio* correlate precisely with differences in the electrophysiological response patterns by "ATP-best" and "AMP-best" olfactory receptors in the Florida spiny lobster, *Panulirus argus* (Derby *et al.*, 1984; Carr *et al.*, 1986). The identification of receptor types with response spectra that can account for observed behavior provides a mechanistic argument required to augment theoretical ecological considerations.

Ammonia is chemoattractive under some conditions, though this ability depends on the capacity of an organism to assimilate ammonia-nitrogen, or to use ammonia as a cue specific to preferred food. Motile phototropic algae, *Chlamydomonas* (Sjoblad and Frederiksen, 1981), *Dunaliella* (Sjoblad *et al.*, 1978), and *Symbiodinium* (Fitt, 1985), all exhibit positive chemotaxis to ammonia gradients. These organisms use ammonia as preferred substrate for inorganic nitrogen during protein synthesis. Scavenging animals and detritivores also may be found attracted to ammonia. They typically depend on biodegradatory bacteria as a principal source of carbon and nitrogen (Newell, 1965; Fenchel, 1970; Hargrave, 1970; Lopez *et al.*, 1977; Wetzel, 1977). Previous investigators have argued that because ammonia is excreted by all living marine heterotrophs, it might signal the presence of nearby prey (Borroni *et al.*, 1986). However, such a cue would not allow for prey discrimination, for differentiation between predator and prey, or for distinction between living prey and aged carrion. The amino acid to ammonia ratio, on the other hand, provides a continuum on which living and dead prey can both be scaled.

Foragers frequently base their preferences on distinguishing prey taxonomic differences. Evidence shows that odor and taste discriminations often depend on unique blendings of chemical substances (Dethier, 1974; Atema *et al.*, 1980). Feeding suppression may therefore arise as a consequence of mixtures that specify inappropriate prey images, independently of the perceived energy and nutrient properties. This may explain why investigators sometimes find suppressant interactions among amino acids (McLeese, 1970; Allison and Dorsett, 1977; Johnson and Atema, 1986). Sensory mechanisms evolve to economize resource use. It should be possible to combine ecological, evolutionary, and physiological arguments to construct testable hypotheses that predict psychophysical events which influence the chemical perception of food.

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# THE BODY PLAN OF THE CYPHONAUTES LARVA OF BRYOZOANS PREVENTS HIGH CLEARANCE RATES: COMPARISON WITH THE PLUTEUS AND A GROWTH MODEL

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## ABSTRACT

The cyphonautes larva of bryozoans is anomalous among ciliary suspension feeders with upstream particle capture. This is because the length of the ciliated band that produces the feeding current does not increase disproportionately relative to body length during larval development and growth. Comparisons with previous studies of other upstream collectors (mostly the pluteus of the echinoid *Dendraster excentricus* or other echinoplutei) demonstrated a striking deficiency in feeding capabilities of the cyphonautes. (1) In comparison to the pluteus the ciliated band generating the feeding current of the cyphonautes was short both absolutely and relative to larval body size as protein. (2) During larval growth, the length of the ciliated band of the cyphonautes decreased relative to protein in the whole body whereas the length of ciliated band of the pluteus was nearly isometric with body protein. (3) The ratio of metabolic capacity (by electron transport system assay) to protein content was similar for the advanced stage pluteus and cyphonautes. If respiratory rate is proportional to metabolic capacity in these larvae, then the cyphonautes does not compensate for low feeding capacity by reduced respiratory rate. (4) The velocity of the current across the ciliated band and the length of lateral cilia was similar for the cyphonautes and pluteus. Therefore, maximum clearance rates per unit length of ciliated band were not unusually high for the cyphonautes. (5) The cyphonautes was inferior in rate of capture of small (2 $\mu$m) spheres relative to 10 $\mu$m spheres in comparison to plutei feeding on the same suspension. (6) The pluteus and cyphonautes were similar in the maximum sizes of spheres ingested. (7) In two experiments the cyphonautes was more selective of spheres flavored by incubation with the alga *Dunaliella tertiolecta* than was the pluteus. (8) Different patterns of allometric growth of ciliated bands were incorporated in a growth model based on the difference between allometry of gain in organic carbon through feeding and loss of organic carbon through respiration. The model predicted that a cyphonautes form required a much greater concentration of food than a pluteus form to gain the same organic material in the same time. Thus the cyphonautes proved inferior to the pluteus in quantitative measures of capacity for suspension feeding. The highly conservative differences in larval body plans result in differing capabilities for feeding that are likely to influence larval growth rates and the evolution of life histories.

## INTRODUCTION

The cyphonautes is the only known feeding larval form of bryozoans. The cyphonautes creates a feeding current with a band of lateral cilia much like the lateral cilia

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of adult bryozoans, other larval and adult lophophorates and the larvae of echinoderms and enteropneusts (Atkins, 1955; Strathmann, 1971, 1973, 1982; Strathmann *et al.*, 1972; Strathmann and Bonar, 1976; Winston, 1978; McEdward, 1984, 1986b). In other animals with this arrangement of cilia and upstream capture of particles, the ciliary band increases disproportionately as the animal grows, with loops of the band increasing in number or length. In contrast, the two bands of lateral cilia in the cyphonautes remain simple curved bands; length of the bands of lateral cilia increases in proportion to body length rather than surface area or tissue volume. The cyphonautes has unusual features that might affect quantitative measures of feeding capability. The bands of lateral cilia are within a mantle cavity (Atkins, 1955) and particles are captured by a sieve of laterofrontal cilia upstream from the lateral cilia (Strathmann and McEdward, 1986). Therefore we examined several features of the cyphonautes for comparison with the better known pluteus larvae of echinoids to answer the following questions. (1) Does the protein content or metabolic rate of the cyphonautes indicate an unusually low quantity of tissue for a larva of its size and thus account for the short band of lateral cilia? (2) Do velocities of particles crossing the bands of lateral cilia indicate faster feeding currents and hence higher clearance rates per unit length of ciliary band? (3) Are the cyphonautes larvae relatively more efficient than plutei at retaining small particles? (4) Could the cyphonautes larvae capture unusually large particles relative to their body size?

The answers to these questions supported predictions based on comparative morphology and indicated that the cyphonautes larval form is not well designed for high rates of ingestion from low concentrations of food. Implications for growth were derived from a model in which allometric changes in uptake of organic carbon were predicted from allometry of ciliated band length and in which allometric changes in losses of organic carbon were predicted from allometry of respiratory capacity. The results predict that cyphonautes larvae require much higher concentrations of food than do plutei to grow at the same rate. Slow larval growth as a consequence of larval body plan may have influenced the evolution of bryozoan life histories.

## MATERIALS AND METHODS

Eggs and earliest stage cyphonautes larvae were obtained at Friday Harbor, Washington, by letting colonies of *Membranipora membranacea* spawn into seawater with 0.0001 *M* EDTA (methods from C. Reed, pers. comm.). Cyphonautes larvae at more advanced stages were collected from the plankton. Larvae were maintained in the laboratory within a few degrees of ambient sea temperature, which ranged from 8°C in early spring to 13°C in late summer. The cyphonautes larvae from the plankton could not be identified to species with certainty but were most likely *Membranipora membranacea*. Those that metamorphosed to ancestrulae could be identified as *Membranipora*, and the most abundant *Membranipora* species near the San Juan Islands is presently thought to be *M. membranacea*.

The plutei of the sand dollar *Dendraster excentricus*, used in feeding experiments for comparison with cyphonautes larvae, were in most cases reared from eggs and sperm in the laboratory by methods described by Strathmann (1971). Those in the experiments with 5 and 10 $\mu$m spheres were obtained from the plankton. Other larval echinoderms, ophioplutei and bipinnariae, were obtained from the plankton.

Dimensions of the larval body were obtained by three-dimensional digitization as described by McEdward (1984, 1985). Body length and apical height were defined as shown in Figure 1. The measured ciliated band length was doubled to account for the two ciliated ridges. Surface area was taken as twice the projected area of the body outline in lateral view. Relative volume was obtained as the product of maximum thickness (from valve to valve in region X, Fig. 1) and projected area.

Exponents of the general allometric equation ($Y = aX^b$) were calculated by Bartlett's nonparametric method (Simpson *et al.*, 1960). The 95% confidence intervals were calculated empirically by nonparametric bootstrapping techniques (Efron, 1979; Diaconis and Efron, 1983). If the isometric exponent fell outside the confidence interval then the difference between the calculated exponent and the isometric exponent was statistically significant.

Protein content was measured by using the Coomassie brilliant blue G-250 dye binding assay method of Bradford (1976) and Sedmak and Grossberg (1977). Electron transport system (ETS) activity was measured using the methods of Owens and King (1975). The artificial electron acceptor in this reaction is 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT). Aqueous extracts of larvae were prepared according to the methods of McEdward (1984).

Lengths of cilia were measured from cyphonautes larvae taken from a single sample during September 1985. Larvae were sorted live, relaxed with isosmotic $MgCl_2$, and then killed with formalin highly diluted with seawater. Because many cilia lay at an angle to the plane of focus, only the cilium that appeared to be longest was measured for each larva. Because many cilia approached the longest observed in each larva, picking the apparently longest cilium for each larva could only slightly overestimate the length of cilia. Poor orientation of many cilia would have resulted in an underestimate of cilia lengths if the mean of a random sample had been taken. Precision for cilium lengths is about ±1 μm. Lengths of the locomotory (coronal) cilia were the same on anterior and posterior loops of the corona.

Films were taken with a high speed 16-mm cinecamera (Redlake Locam) at about 100 or 60 frames/s. A timing light placed a spot on the margin of the film every 0.01 s for more exact calculation of the time between frames. Larvae were held for observation in a *cul de sac* formed by pieces of broken coverglass between the cover glass and slide. Because the cilia generating the feeding current are within the mantle cavity and currents from the locomotory coronal band dominate the flow outside the mantle cavity, we were not concerned with drag from the walls of the glass cage. The velocity measurements were made on a temperature-controlled stage (Cloney *et al.*, 1970) with the slide cooled to about 12°C. The larvae had been maintained in the laboratory at about 10°C.

The particles used to test efficiency of capture of small particles were the polystyrene divinylbenzene spheres that are used in calibrating electronic particle counters. The spheres were suspended in seawater, dissociated by sonication in a water bath, and counted in a hemacytometer before final dilution to test concentrations. Larvae were introduced to a gently stirred suspension with known concentrations of particles of two sizes. After 12 minutes feeding was terminated by preservation in formalin buffered with $CaCO_3$. The gut contents were counted with differential interference contrast optics. The relative rates of particle capture are an estimate of relative efficiency of capture, but because transfer of larvae into the particle suspension disturbs feeding for an undetermined and probably variable time, the absolute rates of capture are not maximum feeding rates.

Polystyrene divinylbenzene spheres were also used to test capacity for qualitative selection of particles. For this test spheres of each size were incubated overnight with either seawater or with algal cells (*Dunaliella tertiolecta* or *Thalassiosira weissflogii*) that had been centrifuged and resuspended in seawater. Larvae were then introduced to suspensions in which one size of particle had been incubated with algae and the other with seawater only. For a more detailed description of this method, see Rassoulzadegan *et al.* (1984).

Spheres of another polymer (trade name Sephadex) were used to test for maximum sizes ingested. These spheres ranged from ~20 to 60 μm in diameter. The

TABLE I

*Larval dimensions for cyphonautes larvae of four sizes*

| Stage<br>n | 1<br>5 | 2<br>16 | 3<br>5 | 4<br>10 |
|---|---|---|---|---|
| Body length (μm) | 155 (±10) | 341 (±5) | 526 (±15) | 649 (±9) |
| Apical height (μm) | 138 (±9) | 267 (±4) | 329 (±7) | 394 (±5) |
| Height/length | 0.90 (±0.03) | 0.79 (±0.01) | 0.62 (±0.01) | 0.61 (±0.00) |
| Thickness (μm) | 43 (±2) | 68 (±5) | 113 (±5) | 143 (±4) |
| Band length (μm) | 123 (±7) | 361.00 (±13) | 577 (±31) | 644 (±24) |
| Surface area ($10^4$ $\mu m^2$) | 3.6 (±0.4) | 14.4 (±0.4) | 28.8 (±1.2) | 43.2 (±1.2) |
| Relative volume | 8.0 (±1.1) | 49.9 (±4.2) | 163.9 (±13.9) | 308.2 (±10.2) |

Mean values (±SE).

spheres were soaked in seawater with *Dunaliella tertiolecta* for four hours; the algal suspension was decanted twice, and the spheres were soaked for another hour in seawater. The larvae were introduced to the suspension for 19 minutes and then preserved in buffered formalin. The diameters of spheres in the guts were measured with an ocular micrometer. To check for preservation artifacts, we compared fresh and formalin preserved subsamples of the stock suspension. Preservation and storage for more than a month had no effect on the size frequency distribution of the spheres.

For a model of growth based on estimated stage specific gains and losses of organic carbon, the coefficients and exponents of allometric equations were obtained by a model I regression (Sokal and Rohlf, 1981) through the logarithms of mean values for each stage. For isometric relationships the coefficient was the mean of ratios of values.

## RESULTS

### *Ciliary band length relative to body size and shape*

The cyphonautes larvae remained roughly triangular throughout growth, but the shape of the triangle changed. Apical height increased slower than body length (Table II). The steady decrease in the height:length ratio (Table I) resulted in longer but squatter larvae in later stages (Figs. 1, 2). Since thickness changed in proportion to body length, surface area increased more slowly with respect to volume than would be expected if later stages were geometrically similar to early larvae (Table II).

The cyphonautes has several bands and groups of cilia. The bands that generate the feeding current through the mantle cavity are the lateral cilia on a pair of ridges separating the upstream (inhalant) and downstream (exhalant) portions of the mantle cavity (Figs. 1, 2). Hereafter "band length" refers to the sum of the lengths of the two ridges because this is the total length of the bands of lateral cilia that generate the feeding current.

Band length of the cyphonautes was nearly isometric with body length. Mean band lengths and mean body lengths were 123 μm and 155 μm for the smallest measured size category and 644 μm and 649 μm for the largest (Table I). Ciliated band length increased in proportion to body length and in proportion to the linear component of volume (Table II).

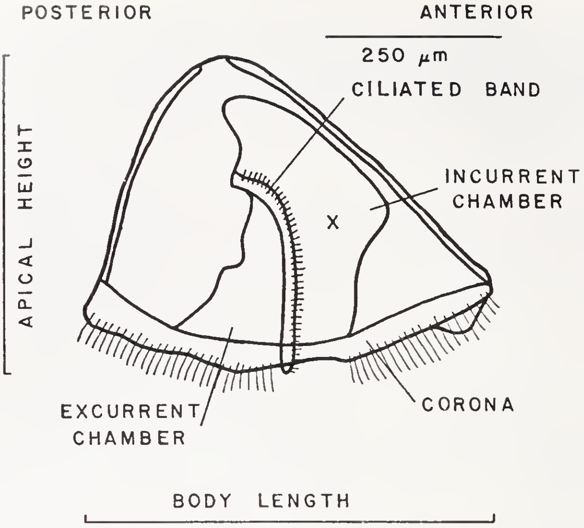

FIGURE 1. Positions of measurements on the cyphonautes.

To compare the cyphonautes with the pluteus we used the protein content of the larvae as a measure of larval size. Protein content of the larva increased in proportion to the cube of body length: 79 ng for a cyphonautes 341 μm long ($n = 2$) and 610 ng for a cyphonautes 649 μm long ($n = 3$). Protein content also increased in proportion to larval volume (Table II). Since band length did not increase relative to body length or volume, the band length decreased relative to protein content of the whole cyphonautes during larval growth (Table II). This was in contrast to the pluteus of *D. excentricus,* in which the ciliated band increased in proportion to larval volume, protein content, and ETS activity of the whole larva during development from the 4 to 8 armed stage (McEdward, 1984). In this pluteus, the protein content increased relative to ciliated band length only during the 8 armed stage, when the rudiments of postmetamorphic juvenile structures were developing.

If larval tissue increases relative to band length, then do metabolic needs increase relative to clearance rate? The ETS assay yielded 1.75 nmols INT $h^{-1}$ $larva^{-1}$ for the

TABLE II

*Exponents (b) of the general allometric equation ($Y = aX^b$)*

| Y | X | b | 95% confidence interval | Isometric b |
|---|---|---|---|---|
| Apical height | Body length | 0.64 | 0.57–0.71 | 1.00 |
| Thickness | Body length | 0.93 | 0.78–1.10 | 1.00 |
| Band length | Body length | 1.03 | 0.94–1.10 | 1.00 |
| Protein | Body length | 2.91 | 2.38–3.72 | 3.00 |
| Protein | Volume | 0.99 | 0.68–1.34 | 1.00 |
| Surface area | Volume | 0.58 | 0.53–0.64 | 0.67 |
| Band length | Volume | 0.37 | 0.32–0.41 | 0.33 |
| Band length | $Volume^{0.33}$ | 1.11 | 0.99–1.25 | 1.00 |
| Band length | Protein | 0.39 | 0.21–0.60 | 1.00 |

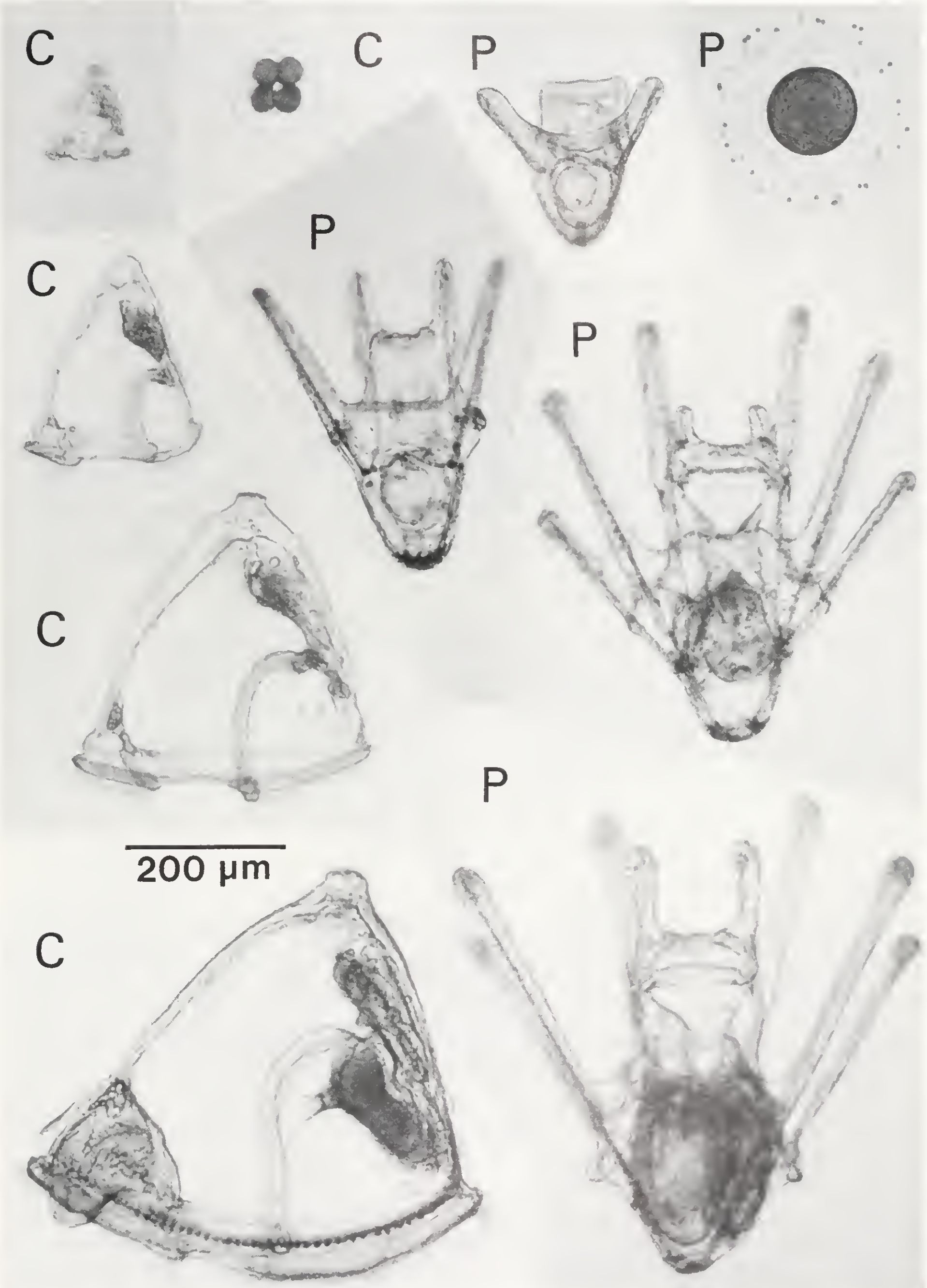

FIGURE 2. Change in size and form of a developing cyphonautes (C) of *Membranipora* and developing pluteus (P) of *Dendraster excentricus*. All embryos and larvae to the same scale.

TABLE III

*Lengths of cilia and larvae in μm*

| Length of larva along open edge | Lateral cilia | Coronal cilia |
|---|---|---|
| 320 | 21 | 38 |
| 330 | 20 | 40 |
| 470 | 21 | 48 |
| 540 | 19 | 57 |
| 600 | 21 | 57 |
| | mean 20 | |

late stage cyphonautes, whose protein content was 610 ng larva$^{-1}$ and body length was 649 μm. This ratio of metabolic capacity to protein was similar to the ratio for late stage plutei of *D. excentricus* (protein 600 ng larva$^{-1}$; ETS 1.44 nmols INT h$^{-1}$ larva$^{-1}$; larval length 699 μm) (McEdward, 1984). Metabolic capacity is likely to be approximately proportional to metabolic rates. If it is, low metabolic rate does not compensate for the low clearance rate of the cyphonautes.

## *Increase in length of cilia and larvae*

Coronal cilia increased in length as the larva increased in length, but length of lateral cilia remained constant (Table III). These cilia lengths suggest an increase in volume flow of water per unit length of the locomotory coronal band as the larvae increase in size but little if any increase in volume flow per unit length of the food capturing ciliated ridge as larval size increases. Cilia lengths of food collecting bands of *D. excentricus* are 20 to 25 μm long and do not increase during larval development (McEdward, 1984). The cilia producing the feeding currents are therefore similar in length and in constancy of length in both larval forms.

## *Particle velocities at the bands of lateral cilia*

To compare velocities of feeding currents of the cyphonautes to previous measurements on the pluteus, we trapped cyphonautes larvae and filmed the passage of 5 μm spheres between the two bands of lateral cilia. Under the conditions of observation, the cyphonautes larvae retained and ate few particles and thus had a low clearance rate, but they maintained a fast current through the mantle cavity. We did not include any particle tracks in which more than two frames elapsed as the particle crossed the band because particles were sometimes arrested on the upstream side of the band and our object was to use velocities of particles as indicators of water velocity past the bands of lateral cilia. Some particles crossed the band in a single interval between frames (1/60 s) with a mean velocity of 1.7 mm/s ($n = 16$). If currents were only intermittently fast enough to carry particles across the band in one frame interval, this would be a biased estimate of velocity of the feeding current, but estimates with more frames may have included particles momentarily detained by latero-frontal cilia or other cilia. For particles crossing the band in two intervals between frames, the mean velocity upstream was 1.0 mm/s, and the mean velocity downstream was 1.4 mm/s ($n = 38$). The upstream velocity was significantly lower than the downstream velocity (Wilcoxon matched pairs signed ranks test, $P < 0.005$). Some particles crossed in two intervals, but the midpoint position was obscured; their mean velocity was 1.3 mm/s ($n = 22$). The grand mean was 1.3 mm/s ($n = 76$).

TABLE IV

*Relative rates of ingestion of spheres of 10 μm and 2 μm diameter*

| | Number of larvae | Total 10 μm ingested | Total 2 μm ingested | Ratio clearance rates (10 μm/2 μm) |
|---|---|---|---|---|
| *Cyphonautes | | | | |
| 490 to 480 μm length | 6 | 76 | 8 | 57 |
| **Dendraster excentricus* | | | | |
| 4 armed pluteus | 20 | 567 | 205 | 16.6 |
| **Cyphonautes | | | | |
| 270 to 490 μm length | 9 | 45 | 5 | 90 |
| ***Bipinnaria* | 1 | 100 | 35 | 29 |
| ***Cyphonautes | | | | |
| 260 to 430 μm length | 19 | 117 | 37 | 32 |
| ****Dendraster excentricus* | | | | |
| 6 armed pluteus | 20 | 757 | 280 | 27 |

* Larvae with 30/μl of 2 μm and 5/μl of 10 μm spheres for 12 min.

** Larvae with 20/μl of 2 μm and 2/μl of 10 μm spheres for 15 min.

*** Larvae with 30/μl of 2 μm and 3/μl of 10 μm spheres for 10 min and 2 μm spheres flavored with *Thalassiosira weissflogii.*

The proportions of 10 μm and 2 μm spheres ingested were significantly different from the proportions in suspension (G test, $P < 0.001$). Larvae with the same number of asterisks were in the same suspension.

Overall, the velocity of currents past the lateral cilia of the cyphonautes larva was similar to velocities of 1.3 and 1.7 mm/s past the ciliated band of plutei of *Lytechinus pictus* at room temperature (Strathmann *et al.*, 1972; Emlet, 1983). The feeding current of the cyphonautes was as fast or perhaps slightly faster than the current of plutei. Faster currents do not compensate for the short ciliated band of the cyphonautes.

### *Capture efficiency for small particles*

Are cyphonautes larvae exceptionally efficient in retaining small particles? We compared the relative rates of ingestion of 10 μm and 2 μm spheres by cyphonautes larvae and by plutei of *Dendraster excentricus* and a bipinnaria (Table IV). The cyphonautes larvae were never much better and usually worse than the echinoderm larvae in the retention of the smaller spheres. Cyphonautes larvae retained the 2 μm spheres at much lower rates than 10 μm spheres even when the 2 μm spheres were flavored by previous incubation with a suspension of algal cells (Table IV). Capture of smaller food does not compensate for the lower clearance rates of the cyphonautes.

### *Limits on the maximum size of particles captured*

Could cyphonautes larvae capture and ingest larger particles than those taken by other larvae with upstream capture? To determine the maximum size of particle that could be ingested, we fed echinoplutei, ophioplutei, and cyphonautes larvae a suspension of spheres for which the mean size was near the maximum size that the larvae could ingest (Table V). A sample of 50 spheres from the suspension (first row of Table V) is included for comparison with spheres ingested by the larvae (subsequent rows). Advanced stages of the cyphonautes and plutei with four to eight arms captured particles of about the same mean size. The mean size for the largest 10% of particles ingested was also similar for the advanced stage cyphonautes and for plutei. Sizes of particles ingested increased with larval size for each type of larva tested, but sizes were comparable for larvae of similar lengths. (The mean midline lengths of 4 armed *D.*

Table V

*Maximum size of spheres ingested by larvae feeding in the same suspension for 19 minutes*

| | Number larvae | Spheres counted | Mean ± S.D. μm | Range μm | Mean of largest 10% ingested |
|---|---|---|---|---|---|
| Suspension sample | — | 50 | 45.1 ± 11.8 | 22.1–70.7 | — |
| Cyphonautes<br>330–400 μm length | 6 | 8 | 28.2 ± 2.6 | 24.3–33.5 | 33.2 |
| Cyphonautes<br>460–580 μm length | 10 | 30 | 31.1 ± 5.6 | 18.8–46.4 | 42.0 |
| *Dendraster excentricus*<br>4 armed pluteus | 10 | 36 | 30.1 ± 4.4 | 22.1–38.7 | 38.2 |
| *Dendraster excentricus*<br>8 armed pluteus | 10 | 62 | 31.6 ± 4.8 | 21.0–43.1 | 40.6 |
| Ophiopluteus<br>6 armed | 3 | 10 | 28.2 ± 4.1 | 21.0–34.3 | 34.3 |
| Ophiopluteus<br>8 armed | 7 | 39 | 30.4 ± 6.9 | 12.1–44.2 | 42.3 |

*excentricus* and 6 armed ophioplutei were 280 μm and 300 μm. The mean midline lengths of the 8 armed plutei were 330 μm.) Capture of larger food does not compensate for the low clearance rates of the cyphonautes.

Does the width of the mantle cavity ultimately limit the size of particles that can be captured by the cyphonautes? No particles this large were ingested in this experiment. Near the center of the upstream side of the mantle cavity (point X in Fig. 1) the depth of the mantle cavity is 143 μm in a cyphonautes larva of 649 μm body length (Table I), but the ingested particles were less than 50 μm. Either the maximum width of the incurrent path is much narrower elsewhere, or the width of incurrent openings greatly overestimates maximum sizes ingested.

### *Selection of particles on the basis of quality rather than size*

Is the cyphonautes more discriminating than other larvae with upstream retention of particles? Larvae were fed a suspension of 5 and 10 μm diameter spheres in equal concentration but with spheres of one size flavored by incubation with cells of *Dunaliella tertiolecta.* All the larvae tested ingested 10 μm spheres at higher rates than 5 μm spheres, but the preferential ingestion of 10 μm spheres was greater when they were flavored than when the 5 μm spheres were flavored (Table VI). In these experiments the effect of flavoring on relative ingestion rates was greater for the cyphonautes than for the echinoderm larvae. Further experiments would be needed to confirm the generality of this result because only one algal species was tested and the effect on ingestion rates by the echinoderm larvae was not pronounced. In contrast, in similar experiments by Rassoulzadegan *et al.* (1984) on larvae of *D. excentricus* the effect of a flavoring from a culture of algal cells was greater and was sufficient to reverse preference for 5 and 10 μm spheres. Differences in discrimination may exist, but establishing both the existence of differences in selectivity and the consequences of such differences for larvae under natural conditions will require a more extensive set of comparisons.

### *A model relating allometric change in form to growth rates*

An important consequence of larval feeding is growth, because faster growth results in a shorter planktonic period and presumably therefore less loss of larvae

TABLE VI

*Selection of particles on the basis of presence or absence of flavor acquired by incubation with* Dunaliella tertiolecta

| | Number of larvae | Flavored bead | Total 10 μm ingested | Total 5 μm ingested | Ratio of ingestion rates (10 μm/ 5 μm) |
|---|---|---|---|---|---|
| *Cyphonautes | 18 | 5 μm | 55 | 36 | 1.5 |
| **Cyphonautes | 24 | 10 μm | 141 | 51 | 2.8 |
| 290 to 590 um length | | | | | |
| **D. excentricus* | 1 | 5 μm | 78 | 38 | 2.1 |
| ***D. excentricus* | 1 | 10 μm | 90 | 31 | 2.9 |
| 8 armed pluteus | | | | | |
| *Bipinnaria | 2 | 5 μm | 31 | 13 | 2.4 |
| **Bipinnaria | 4 | 10 μm | 130 | 37 | 3.5 |
| ***Cyphonautes | 25 | 5 μm | 198 | 175 | 1.1 |
| ****Cyphonautes | 26 | 10 μm | 245 | 58 | 4.2 |
| 500 to 630 um length | | | | | |
| ****D. excentricus* | 7 | 5 μm | 704 | 419 | 1.7 |
| *****D. excentricus* | 7 | 10 μm | 654 | 288 | 2.3 |
| 8 armed pluteus | | | | | |

* and **: Larvae with 5/μl of 5 μm and 5/μl of 10 μm spheres for 12.5 min.
*** and ****: Larvae with 5/μl of 5 μm and 5/μl of 10 μm spheres for 10 min.
Larvae with the same number of asterisks were in the same suspension.

through transport or predation. Can observations on ciliated band length (here proportional to maximum clearance rate) and metabolic capacity (assumed proportional to losses from respiration) be converted to predictions about larval growth at different concentrations of food? We expect allometric changes in feeding capacity to be a major determinant of duration of the planktonic period from first feeding to competence for settling or metamorphosis but the relationship is not simple. Here we explore some of the simplest possible models for the relation between allometry of band length and duration of the larval period.

We wish to explore implications of allometric changes in form for duration of the larval period. Our reasoning is similar to that of Pütter (1920) and von Bertalanffy (1957) except that we are more willing to sacrifice accurate description of growth by fitted curves (Ricker, 1979) for the sake of analysis of effects of form. For this reason we have used the general form of their model of growth in organic material (w) with time (t) as

$$dw/dt = Bw^b - Aw^a \tag{1}$$

but without their restrictive assumptions about the value of the exponents b and a. This permits comparisons based on observed allometric relationships of different larval forms.

If clearance rates are approximately proportional to lengths of ciliated bands and if respiratory rates are approximately proportional to capacity of the electron transport system then rate of growth can be modelled as the difference between two allometric equations as in equation (1) where $Bw^b$ is rate of intake of materials and $Aw^a$ is rate of loss of materials. Integrating this expression from the starting organic content to the organic content when the larva is competent to metamorphose gives the minimum planktonic period (T).

$$T = \int_{w_0}^{w_1} dw/(Bw^b - Aw^a) \tag{2}$$

This can be solved by several substitutions. First let $u = (A/B)w^{a-b}$ and rearrange to get

$$T = C\int_{u_0}^{u_1} (u^c du)/[u(1 - u)]$$

where $C = [(B/A)^{(1-b)/(a-b)}]/[B(a - b)]$ and $c = (1 - b)/(a - b)$ and then let $v = u/u_1$ and $v' = u/u_0$ and rearrange to get

$$T/C = u_1^{(c-1)} \int_0^1 [u_1 v^{(c-1)} dv]/(1 - u_1 v) - u_0^{(c-1)} \int_0^1 [u_0 v'^{(c-1)} dv']/(1 - u_0 v') \quad (3)$$

The solution to equation (3) is in Gradshteyn and Ryzhik (1980, p. 286, #3.197.3) in terms of a Beta function and Gauss' hypergeometric function

$$T/C = B(c, 1)[u_1^c F(1, c; c + 1; u_1) - u_0^c F(1, c; c + 1; u_0)]$$

when $c > 0$. This expression simplifies to

$$T = C[(A/B)(w_1)^{a-b}]^c \sum_{n=0}^{\infty} [(A/B)w_1^{a-b}]^n/(c + n)$$

$$- C[(A/B)(w_0)^{a-b}]^c \sum_{n=0}^{\infty} [(A/B)w_0^{a-b}]^n/(c + n) \quad (4)$$

The condition that $(1 - b)/(a - b) > 0$ limits the range of values for the allometric exponents for this method of relating larval form to planktonic period.

A special case of interest is $c = 1$ because this occurs when $a = 1$, and the allometric exponent for losses through respiration and excretion is likely to be close to 1 for many ciliated larvae. When $c = 1$, equation (4) simplifies further (Gradshteyn and Ryzhik, 1980, p. 44, 1.513.4) to become

$$T = [1/(A(1 - b))] \ln [(B - Aw_0^{1-b})/(B - Aw_1^{1-b})] \quad (5)$$

When the exponents a and b both equal 1, the result is simply

$$T = [1/(B - A)] \ln (w_1/w_0) \quad (6)$$

Equations (4), (5), and (6) apply under restricted conditions. There must be growth throughout the range of sizes. Suspended food must be ingested and assimilated at rates proportional to the length of the ciliated band that produces the feeding current. Material must be lost from the body at a rate dependent on size but independent of concentration of food. A realistic and quantitative prediction would only be possible for the range of concentrations of food that results in nearly constant respiration and clearance rates. Both respiration and clearance rates vary with food concentration for a variety of suspension feeders. Several types of echinoderm larvae reduce their clearance rates above about 1000 cells/ml of *Dunaliella* spp. (Strathmann, 1971; Lucas, 1982). Concentrations of food could also affect respiration (Fenaux *et al.*, 1980) and assimilation by these larvae. The situation is complicated for *Dendraster excentricus* by the development of longer arms (and therefore a longer ciliated band) at lower concentrations of food (I. Boidron-Metairon, pers. comm.). The following calculations are therefore for gross comparisons between the cyphonautes and pluteus forms.

Application of the model to the cyphonautes of *Membranipora* and pluteus of *Dendraster excentricus* requires additional assumptions about measurements and conversion factors. Table VII gives mean values for body protein, the electron transport system assay, and length of ciliated band. Table VIII gives coefficients and expo-

TABLE VII

*Measures of size and metabolic capacity for the cyphonautes of* Membranipora *and pluteus of* Dendraster excentricus

| Larva | Cyphonautes | | | Pluteus | | | |
|---|---|---|---|---|---|---|---|
| Stage | New | Small | Advanced | 4 arm | 6 arm | 8 arm | 8 arm with rudiment |
| Protein ($\mu$g) | 0.013* | 0.078 | 0.61 | 0.079 | 0.123 | 0.169 | 0.599 |
| ETS (nmol INT/h) | — | — | 1.75 | 0.093 | 0.21 | 0.22 | 1.44 |
| Ciliated band (mm) | 0.12 | 0.36 | 0.64 | 1.45 | 2.50 | 4.20 | 6.90 |

* Protein value from egg.

nents for the allometry of rates of gain and loss in relation to body organic content in terms of organic carbon. To get from Table VII to Table VIII we made the following assumptions and calculations.

Organic carbon in the body was assumed to be 0.75 times the protein, a conversion taken from an assumed ratio of 17.8% nitrogen in the protein in the whole body and from a ratio of carbon to nitrogen of 4.2 for zooplankton (mean among phyla) (Omori and Ikeda, 1984). This conversion would underestimate organic carbon in later stages if later stage larvae store more fat. The measured protein content per egg was assumed to be equal to the protein in the early stage cyphonautes. Protein probably declines so that organic content of the early cyphonautes was overestimated. As a result growth of the cyphonautes was underestimated, and this biases the comparison in favor of the cyphonautes.

We ignored excretory losses and assumed that the respiratory rate was directly proportional to the ETS assay, which was assumed to be proportional to the protein measurement. For comparison of cyphonautes and pluteus we used the mean of (ETS)/(protein) for the five available points for both types of larvae (Table VII). We assumed 2 nmol INT = 1 nmol $O_2$ (Immers and Runnstrom, 1960; DeVincentiis *et al.,* 1966), a value within the range observed for other zooplankton though toward the lower end of the range (Omori and Ikeda, 1984), and for conversion to organic carbon assumed a respiratory quotient of 1. For the pluteus we also used the regression through the mean values for the four stages. The coefficients and exponents assumed for isometric and allometric rates of loss are in Table VIII.

To convert ciliated band length to a rate of clearance and assimilation, we assumed that the volume flow per mm of band was a 20 $\mu$m length of cilium times half a tip velocity of 1.7 mm/s times 1 mm and that the assimilation efficiency was 60%, a value within the calculated range for larvae of *Mytilus edulis* (Sprung, 1984) and above some estimates for gastropod veligers (Pechenik, 1980). The coefficient B in equation (1) was partitioned into the product of $B_1$ and the concentration of food (F). For comparison of cyphonautes and pluteus we assumed allometric relationships between ciliated band length and body organic content. For the pluteus we also tried an isometric relationship between band length and body organic content because this relationship is approximately isometric for much of larval development (McEdward, 1984). Coefficients ($B_1$) and exponents (b) for assumed allometric and isometric relations are in Table VIII.

With these values and equations (4), (5), and (6), we calculated the concentration of food required for growth through the feeding larval stages in three weeks. The cyphonautes required about ten times the concentration of food required by the pluteus (Table VIII). This result was not changed when rates of loss by the pluteus were

TABLE VIII

*Results of a growth model predicting concentration of food required for growth from organic content $W_1$ to $W_2$ in μg C in 21 days*

| Larva | $B_1$ | b | A | a | $W_1$ | $W_2$ | F |
|---|---|---|---|---|---|---|---|
| | | | Observed growth | | | | |
| Cyphonautes | 0.868 | 0.424 | 0.364 | 1 | 0.00945 | 0.458 | 0.27 |
| Pluteus | 12.3 | 0.730 | 0.364 | 1 | 0.0592 | 0.449 | 0.025 |
| Pluteus | 12.3 | 0.730 | 0.584 | 1.321 | 0.0592 | 0.449 | 0.026 |
| Pluteus | 22.1 | 1 | 0.364 | 1 | 0.0592 | 0.449 | 0.021 |
| | | | Same growth | | | | |
| Cyphonautes | 0.868 | 0.424 | 0.364 | 1 | 0.045 | 0.45 | 0.27 |
| Pluteus | 12.3 | 0.730 | 0.364 | 1 | 0.045 | 0.45 | 0.026 |

The growth equation is $dW/dt = B_1FW^b - AW^a$ with F the concentration of food in μg C/ml, $B_1$ and b estimated from ciliated band length, and A and a estimated from metabolic capacity. See text for the estimates of allometric shifts in loss and gain and other assumptions.

assumed to be allometric or when rates of gain by the pluteus were assumed to be isometric, though such changes did produce changes in shapes of growth curves, as expected (see Fletcher, 1975). The result was also not changed when the same interval of growth was assumed for both cyphonautes and pluteus. When concentrations of food are limiting, the cyphonautes should be at a substantial disadvantage relative to the pluteus in rate of growth.

Development times in the model were extremely sensitive to concentration of food, however. This sensitivity is highly unrealistic. Rates of development and growth of plutei of *Dendraster excentricus* do not decrease in proportion to decreased concentrations of phytoplankton (Paulay *et al.*, 1985), and rates of growth and development of larvae of the seastar *Acanthaster planci* are similar in the food poor waters of the Great Barrier Reef and much higher rations in culture (Olson, 1985). These and many other observations indicate that adjustments in rates of loss or gain of organic material with different concentrations of food are important for these larval forms. Because our model lacks compensatory adjustments to different food supplies, the model may exaggerate the predicted differences in food required for similar growth rates.

Ciliated band length and metabolic capacity can be combined for another comparison of the cyphonautes and pluteus forms. Volume of water cleared per volume of oxygen consumed is a measure of feeding efficiency from which one can extrapolate to a concentration of food at which ingestion would equal respiratory losses, a break even point if clearance and respiration rates were constant and assimilation efficiency 100% (Jørgensen, 1966). With conversion factors similar to those for the growth model except for 100% assimilation efficiency, the late stage cyphonautes "broke even" at 130 μg C/l, the late stage pluteus with a rudiment at 10 μg C/l, and the earlier 4 to 8 armed stage plutei at about 3 to 4 μg C/l. Because this calculation assumed that respiration does not decline at low ingestion rates, the predicted concentrations of food required to avert starvation are probably too high. This simpler comparison also predicts a relative disadvantage for the cyphonautes form at low concentrations of food.

Simple models predict large differences in growth of the cyphonautes and pluteus because of differences in the lengths of ciliated bands that produce the feeding currents.

## DISCUSSION

The cyphonautes larva of bryozoans has an anomalously short band of lateral cilia that generate the feeding current. The current velocity at the bands of lateral cilia, the length of lateral cilia, and the relative rates of capture of large and small food particles are similar to those for plutei, which have a much longer ciliated band at a similar body size. The contrast extends to other suspension feeders with upstream capture. The lateral cilia of the cyphonautes are no longer than those of adult bryozoans, phoronids, and brachiopods or those of larval phoronids, brachiopods, enteropneusts, and echinoderms. The cyphonautes does have one feature as yet unobserved in related suspension feeders with upstream capture. It filters particles with a sieve of laterofrontal cilia (Strathmann and McEdward, 1986). However, it appears that no expansion of capabilities is associated with this sieving mechanism. Because the cyphonautes has no apparent means of compensating for its low rate of clearing water of suspended particles, it appears to be at a disadvantage relative to other forms in regard to feeding in waters with low concentrations of food.

Because the cyphonautes larval form occurs in both ctenostome and cheilostome bryozoans, it is probably the ancestral larval form for the class Gymnolaemata (Nielsen, 1971), yet most species in this class now lack a feeding cyphonautes stage. One hypothesis for the scarcity of feeding larvae in the group is that the ancestral larval form had a poor capability for feeding and growth under conditions common in coastal waters. Against this hypothesis is the observation that cyphonautes larvae nevertheless have broad geographic and seasonal occurrence and are common in waters low in phytoplankton. Moreover, the eggs of bryozoans with cyphonautes larvae can be quite small. In the San Juan Islands *Membranipora membranacea* has an egg diameter of about 60 $\mu$m, smaller than that of the local echinoderms (Emlet *et al.*, 1986) and phoronids (Zimmer, 1964; Emig, 1974). Organic content increases with increasing egg size among species with feeding larvae from several phyla (Strathmann and Vedder, 1977), and the protein per egg for *Membranipora membranacea* (0.013 $\mu$g) is less than that for four species of local echinoids (.024–.148 $\mu$g) (McEdward, 1986a). Parental investment per offspring is probably lower for *Membranipora membranacea* with its cyphonautes larva than for co-occuring echinoids and phoronids that use similar larval ciliated bands in larval feeding but have larval forms with apparently greater capacity for clearing particles from suspension. With small eggs and low clearance rate, the larval bryozoan is predicted to have an unusually long planktonic period with associated risks. Yoshioka's (1982) best fit for a planktonic period from field data off Southern California was about four weeks, not extraordinarily long for a larva. We cannot yet resolve these apparent discrepencies.

The combination of poor design for foraging, small initial larval size, and broad geographic occurrence suggests that the cyphonautes larval form has some other redeeming qualities. Any capabilities in feeding that may have been missed in this study would have to be substantial to compensate for the short ciliated band. Another hypothesis is that the cost of slow growth may be low. The only field estimate of mortality rates of the cyphonautes (Yoshioka, 1982) was no lower than those for bivalve veligers and other small larvae (reviewed in Strathmann, 1985), though all of these mortality estimates depended on assumptions about sampling and were subject to large errors. Slow larval growth might be advantageous if there was no advantage to more growth before settlement and if there was an advantage to a long precompetent period, but it is difficult to imagine situations in which these two conditions are commonly met (Strathmann, 1985). Thus possible compensatory benefits from the cyphonautes form are not yet apparent.

A protective shell is not a constraint on length of ciliary band in all larval forms. A shell is combined with an extensible feeding organ in larvae of inarticulate brachiopods, which have separate lateral cilia and upstream capture of food as does cyphonautes, and in larvae of gastropod and bivalve molluscs, which differ in that they capture food downstream using a band of compound cilia. Though the cyphonautes form and short ciliated band are conservative, functional or developmental constraints on this form have not been demonstrated.

In conclusion, the cyphonautes larval form has an anomalously poor capacity for suspension feeding. This is predicted to result in slower growth than in other larvae with upstream capture by cilia when food is scarce. A compensating advantage is not yet apparent.

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# RESPONSE OF GREEN HYDRA TO FEEDING AND STARVATION AT FOUR IRRADIANCES

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## ABSTRACT

The relationship between the productivity of symbiotic algae and growth of the hydra, *Hydra viridissima* (Florida strain), was investigated in hydra maintained at four irradiances (5, 10, 15, and 30 $\mu E \cdot m^{-2} \cdot s^{-1}$) and either fed or starved. Although the productivity of fed hydra increased from 0.3 to 1.15 $\mu g\ C \cdot mg\ protein^{-1} \cdot h^{-1}$ with increase in culture irradiance, there was no significant effect of culture irradiance on population growth or on the protein biomass of individual hydra. The survival of starved hydra was similarly not affected by culture irradiance. Algal-animal biomass parameters changed in response to feeding and culture irradiance. Numbers of symbiotic algae in fed hydra declined with increased culture irradiance. The protein content of hydra starved for 28 days declined to 10% of initial levels. The relative proportion of algal to animal biomass increased in starved hydra as both algal densities and algal cell volumes were almost twice those of fed hydra. Whereas culture irradiance and feeding alter the ratio of algal to animal biomass, growth of this green hydra is only affected by feeding.

## INTRODUCTION

The green hydra, *Hydra viridissima,* maintains *Chlorella*-like algae within its digestive cells. Algal photosynthesis is an important feature of the symbiosis, as photosynthetically fixed carbon is translocated from the algae to the animal host (Muscatine and Lenhoff, 1963; Mews, 1980). Numerous studies show that light sustains green hydra, especially when food is absent or in limited supply. However, the extent to which the productivity of the symbiotic algae influences the growth and survival of green hydra remains unknown. Differences in symbiont productivity can be obtained by maintaining green hydra at a series of low irradiances where photosynthesis is light-limited. Using this approach, our objective was to measure the effect of symbiont productivity on changes in the biomass of green hydra.

Growth rates of fed green and aposymbiotic (=algae-free) hydra maintained in the light showed that light enhances growth of symbiotic hydra, but only when food is limiting (Muscatine and Lenhoff, 1965b). Growth efficiencies derived for green hydra show that the contribution of symbiotic algae is significant, and that this contribution increases with a decrease in feeding frequency in the light (Stiven, 1965a). The nutritional importance of symbiont photosynthetic products is also illustrated by starvation experiments in which survival of symbiotic and aposymbiotic hydra was compared. If sufficiently illuminated, symbiotic hydra survive considerably longer periods of starvation than nonsymbiotic species or aposymbiotic animals (Muscatine and Lenhoff, 1965b; Stiven, 1965b; Kelty and Cook, 1976; Rahat and Reich, 1980).

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Similarly, symbiotic hydra starved in light retain more protein and increase glycogen stores in comparison to aposymbiotic and nonsymbiotic hydra (Cook and Kelty, 1982). Respiratory quotients of symbiotic hydra starved in light indicate that these organisms metabolize mainly carbohydrates, presumed to originate from the symbionts; hydra starved in the dark catabolize mainly fat (Pardy and White, 1977). Light is the key factor influencing the ability of symbiotic hydra to survive starvation; in the dark the algae can represent a significant energetic cost to the hydra (Douglas and Smith, 1983). In these light/dark studies, it is assumed that the productivity of symbiotic algae is responsible for the obtained results, although rates of carbon fixation were not provided.

To examine the contribution of algal productivity to hydra growth, the productivity and growth of green hydra maintained at different irradiances was measured. Since photosynthesis is dependent on irradiance at low photon flux densities, we hypothesized that light may influence the growth and survival of symbiotic hydra cultured under low irradiances. The effect of culture irradiance on the relative proportion of algal to animal biomass of fed green hydra was also measured. Furthermore, although light has been shown to enhance the survival of starved green hydra, it is not known if the total photon flux density available to the algae affects the long-term survival of starving green hydra. We addressed this question by analyzing the survival of starved green hydra acclimated to four different irradiances, and by measuring algal-animal biomass parameters of starved symbiotic hydra.

## Materials and Methods

### *Maintenance of hydra cultures*

Hydra originating from a single clone of *Hydra viridissima* (Florida strain) were used for all experiments. Approximately two years prior to these experiments a symbiosis was established between aposymbiotic hydra and cultured symbionts (NC64A) previously isolated from symbiotic paramecia (*Paramecium bursaria*). Pardy and Muscatine (1973) showed that this association formed a stable endosymbiosis, and that growth rates of these hydra were nearly identical to those of the original stock of green hydra. These hydra were designated F/NC hydra. F/NC stocks were maintained in M solution (Muscatine and Lenhoff, 1965a) in glass dishes at 21°C under continuous light ranging from 25 to 40 $\mu E \cdot m^{-2} \cdot s^{-1}$, PAR (Photosynthetically Active Radiation; 400–700 nm) provided by banks of fluorescent lamps. Hydra were cultured according to the methods of Loomis and Lenhoff (1956). Animals were fed to repletion daily (M–F) with newly hatched *Artemia* sp. nauplii, and were rinsed twice daily with fresh culture solution.

To determine the effect of irradiance on growth and biomass parameters of F/NC hydra, approximately 150 animals from stock culture were placed into each of four glass dishes (28 cm × 18 cm × 5 cm) in about 800 ml M solution. Three dishes were covered with one to three layers of fine mesh plastic screen to reduce irradiance; all dishes were covered by a Plexiglas sheet to minimize evaporation of culture medium. Photon flux density in the four dishes was measured prior to addition of hydra and culture medium with a quantum sensor connected to a Li-Cor Model 185 quantum meter. Average photon flux densities in the four experimental dishes were 30, 15, 10, and 5 $\mu E \cdot m^{-2} \cdot s^{-1}$ (PAR). Experimental dishes were maintained under continuous light at 21°C, and were rotated daily to minimize possible heterogeneity of incident irradiance.

Hydra maintained at the four different irradiances were fed four times weekly with *Artemia* sp. nauplii (M,W,F,Su), and were rinsed daily with M solution. After six months, the animals were subsequently starved for one month to assess the effect of starvation on biomass parameters.

### *Hydra population growth*

Population growth of fed hydra after three months at the experimental irradiances was measured as follows: six replicate groups of three hydra, each with one bud, were selected from each treatment 24 h after feeding. Groups of animals were placed into petri dishes (3.5 cm × 1 cm) containing 5 ml culture solution and covered with screen material to provide similar irradiances as the parent cultures. Hydra were maintained under the same feeding and cleaning conditions as described for parent cultures. The positions of the four groups of dishes were rotated daily; the six replicate dishes at each irradiance were moved as a unit. Hydranths in each dish were counted daily for 8–10 days. Population growth rate constants and doubling times were calculated according to Loomis (1954).

To determine if culture irradiance affected survival of hydra under starvation, fed hydra were placed into individual petri dishes as described above and were starved for more than 80 days. Daily, the number of hydranths in each dish was counted, the culture medium was replaced, and dish positions were rotated.

### *Productivity of hydra*

To measure photosynthesis of symbiotic algae in hydra acclimated to four irradiances, groups of twenty-five hydra (each with one fully developed bud) were incubated with $NaH^{14}CO_3$ ($0.8\ \mu Ci \cdot ml^{-1}$) in 5.0 ml M solution supplemented with 5 m*M* $NaHCO_3$. Hydra maintained on a regime of 4 feedings per week were last fed 24 hours prior to an experiment. All incubations were carried out at 21°C for one hour at 1100–1200 h. The productivity of hydra at different irradiances was estimated by placing groups of hydra in beakers covered with various layers of screen. Replicate groups of hydra in beakers covered with foil were also incubated to correct for any dark fixation of $NaH^{14}CO_3$. Incubation media were sampled at the start of the experiments for total $^{14}C$ activity. At the end of the incubations, hydra were thoroughly rinsed with M solution and then homogenized in distilled water. Aliquots of the homogenate solutions were withdrawn for protein analysis (Lowry *et al.*, 1951) and determination of algal numbers (see methods under hydra biomass parameters). Organic $^{14}C$ retained by green hydra was estimated by analyzing three replicate samples (20 $\mu$l) of homogenized hydra. Samples were acidified by addition of 100 $\mu$l of 0.1 *N* HCl under gentle heat to drive off $^{14}CO_2$. Scintillation fluor (Budget-Solve, Research Products International Corp.; 5 ml) was added, and samples were analyzed in a Packard Tri-Carb liquid scintillation spectrometer (Model 3375).

After correction for dark fixation, the amounts of organic $^{14}C$ retained by hydra were converted to rates of carbon fixation (Vollenweider, 1969). Total $CO_2$ in the incubation medium was calculated from the total alkalinity, measured potentiometrically (Golterman, 1969). Productivity data were fit by linear regression to estimate photosynthetic efficiencies; all correlation coefficients were greater than 0.95.

### *Hydra biomass parameters*

The response of symbiotic hydra to different culture irradiances was quantified using replicate samples of 100 pooled hydra which were analyzed for total protein, number of algae, and algal chlorophyll. Hydra were sampled 24 h after feeding. Sampling of hydra was standardized by selecting individuals with one fully developed bud. As most 30-day starved hydra did not have buds, pooled samples consisted of single individuals. Hydra were homogenized in distilled water with a glass tissue homogenizer. The volume of the homogenate solution was recorded and aliquots removed and frozen for later protein analysis by the method of Lowry *et al.* (1951) using bovine serum albumin as a protein standard. Symbionts were isolated from the remaining homogenate solution and washed in distilled water by repeated centrifugation and resuspension of the algal pellet. The final algal pellet was suspended in a known volume of distilled water and cell concentrations determined by six replicate counts of an aliquot using an A/O Spencer Bright-Line hemacytometer and a Nikon compound microscope at 400×. The total number of symbionts per weight of hydra protein was calculated.

The number of symbionts per hydra digestive cell was estimated by placing groups of 25 hydra into maceration fluid (David, 1973) in a small glass vial. After 10 minutes cells were dissociated by gently tapping the vial on a hard surface. Using phase microscopy at 400×, numbers of algae in 100 randomly selected digestive cells were counted.

Chlorophyll content of freshly isolated symbionts was determined by overnight extraction at 4°C of known numbers of algae in absolute methanol. Absorbances of the supernatants of centrifuged methanol extracts were read in a Beckman DB spectrophotometer at 650 nm and 665 nm. The equations of Holden (1976) were used to calculate the weight of chlorophyll per cell.

Analysis of variance (ANOVA) was used to determine if culture irradiance had a significant effect on biomass parameters of fed hydra. The Least Significant Difference test was applied at the 5% significance level to data sets for which a significant F-ratio was obtained (Sokal and Rohlf, 1969). Due to the low numbers of starved hydra, it was not possible to obtain replicate samples for the biomass parameters and therefore those data could not be analyzed for statistical significance.

Cell volumes of symbionts were determined from measurements of cell diameters obtained using an ocular micrometer at 400×. The mean cell diameter was obtained from measurements of 100 randomly selected algae in hydra homogenized in M solution; algal volumes were calculated assuming spherical cells.

## RESULTS

### *Population growth of fed hydra and survival of starved hydra maintained at four culture irradiances*

Population growth rate constants (k) of fed hydra acclimated to four culture irradiances are given in Table I. There was no significant effect of culture irradiance on k (ANOVA $F_{3,20} = 0.53$; $P = 0.67$), which averaged 0.2238 $\text{day}^{-1}$. Similar results were obtained with a replicate experiment in which k of hydra at the four irradiances averaged 0.2216 $\text{day}^{-1}$. These results show that, with a 4×/week feeding regime, there is no significant difference in population growth of hydra at culture irradiances ranging from 5 to 30 $\mu E \cdot m^{-2} \cdot s^{-1}$.

To determine if culture irradiance had an effect on the survival of symbiotic hydra under starvation, animals maintained at the four culture irradiances for six months with a 4×/week feeding regime were isolated from parent cultures and starved. Some hydra continued to produce buds, even after 20 days without food. Data sets from five-day intervals were analyzed by the general linear models procedure (SAS, 1982). An analysis of covariance using a split-plot design with unreplicated whole plots

TABLE I

*Population growth rate constants (k) and doubling times (T) of F/NC hydra fed 4×/week at 4 culture irradiances*

| Culture irradiance ($\mu E \cdot m^{-2} \cdot s^{-1}$) | k ($day^{-1}$) | T (days) |
|---|---|---|
| 5 | 0.2084 (±.0216)[a] | 3.33 |
| 10 | 0.2258 (±.0094) | 3.07 |
| 15 | 0.2308 (±.0102) | 3.00 |
| 30 | 0.2303 (±.0135) | 3.01 |

[a] n = 6; ±S.E.

(SAS, 1985) was used to determine if culture irradiance had a significant effect on the rate of decline of numbers of hydra. The preceeding 5-day data set was designated as a covariate. Analysis of covariance indicated that there was no significant difference between the decline in hydra populations maintained at the four culture irradiances ($F_3 = 2.16$; $P = 0.102$). Days of starvation had a highly significant effect on the decline of numbers of hydra ($F_1 = 15.70$; $P = 0.0002$). Since culture irradiance did not have a significant effect on the survival of F/NC hydra, the data for the four treatments were pooled and the decline in numbers of hydra as a function of days of starvation described by a second order polynomial equation fit to the combined data by the Proc Reg program (SAS, 1985):

$$\text{Number of hydra surviving} = 0.002406D - 0.000828D^2 + 5.76$$

$$(R = 0.802, n = 72)$$

where D = number of days. Figure 1 shows the decline in the number of hydra with days of starvation. There was no real decline in the number of hydra until Day 40 of starvation, and it took 60 days for the population of hydra to decrease to half the initial numbers.

### *Productivity of hydra*

The productivity of algal symbionts in fed hydra was examined by measuring the amount of $^{14}C$ retained in hydra exposed to different irradiances. It is not clear if the retention of $^{14}C$ is a measure of net or gross production by the symbiotic algae since the extent of $^{14}CO_2$ recycling between animal and algae is unknown. Productivity here refers to the amount of $^{14}C$ retained by the combined algae and animal tissue after a one hour incubation. To determine if photosynthesis was light-limited at the four culture irradiances, the productivity of hydra from each culture irradiance was measured at several irradiances. The productivity of hydra as a function of irradiance (P-I) is shown in Figure 2. The linear rates indicate that photosynthesis of symbionts was limited by light over the range of irradiances examined. Hydra maintained at a culture irradiance of 10 $\mu E \cdot m^{-2} \cdot s^{-1}$ (Fig. 2b) were the most productive hydra at all measured irradiances.

The photosynthetic efficiencies of hydra acclimated to the four culture irradiances (the slopes of the P-I curves in Fig. 2) are given in Figure 3. Photosynthetic efficiency declined with an increase in culture irradiance from 10 to 30 $\mu E \cdot m^{-2} \cdot s^{-1}$, but hydra maintained at 5 $\mu E \cdot m^{-2} \cdot s^{-1}$ were only half as efficient as those maintained at 10 $\mu E \cdot m^{-2} \cdot s^{-1}$ at utilizing the available light for photosynthetic carbon production.

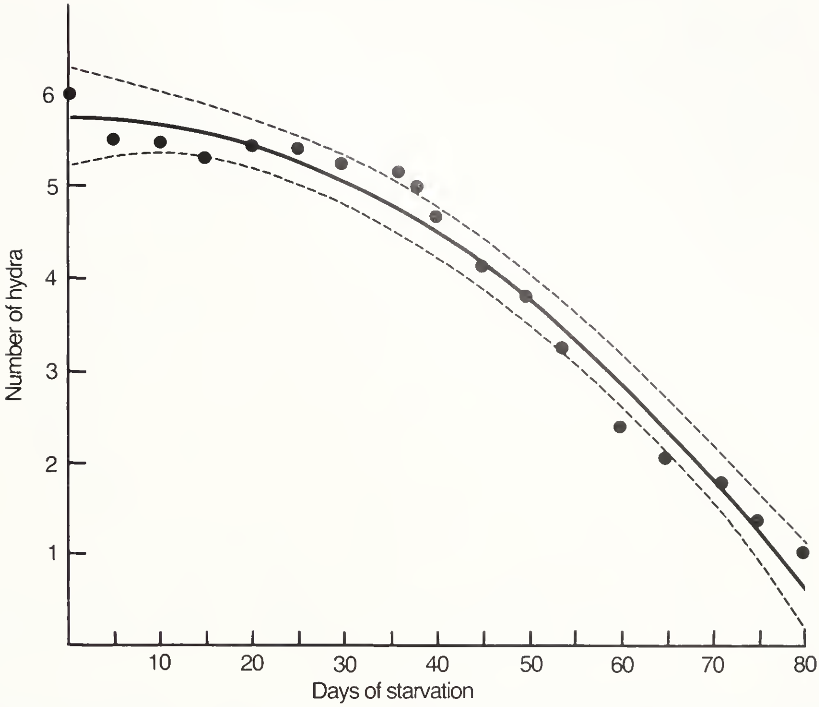

FIGURE 1. Decline in number of hydra with days of starvation. Since the survival of hydra at the four irradiances was not significantly different, the data were pooled and a second order polynomial equation fit to the combined data. Dotted lines represent the 95% confidence intervals for the equation (see text).

The productivity of green hydra at each acclimated culture irradiance ($P_i$) showed that hydra maintained at 30 $\mu E \cdot m^{-2} \cdot s^{-1}$ fixed almost four times the amount of carbon fixed by hydra maintained at 5 $\mu E \cdot m^{-2} \cdot s^{-1}$ (Fig. 4b). Hydra population growth rate constants at each culture irradiance were plotted on the same figure (Fig. 4a) as $P_i$ to compare the two parameters. Although k was not significantly affected by culture irradiance, the curves in Figure 4 show that population growth rates of hydra (Fig. 4a) and hydra productivity (Fig. 4b) follow, in general, the same pattern.

### *Effect of culture irradiance on biomass parameters of fed hydra*

The biomass parameters of fed hydra after one month at four culture irradiances are shown in Figure 5. The protein biomass of fed hydra (Fig. 5a) was not significantly affected by culture irradiance (ANOVA $F_{3,16} = 0.81$; $P = 0.50$). Together with the data in Table I, these results show that culture irradiance does not affect either the growth of individual hydra or that of hydra populations.

The population density of symbionts in fed hydra was affected by culture irradiance (ANOVA $F_{3,16} = 6.36$; $P = 0.005$). Hydra maintained at the highest irradiance contained significantly fewer symbionts than those maintained at the three lower irradiances (Fig. 5b). Similar results were obtained with numbers of symbionts per digestive cell (Fig. 5d). There was a significant effect of culture irradiance on the number of algae per digestive cell (ANOVA $F_{3,8} = 4.71$; $P = 0.03$), but in this case there

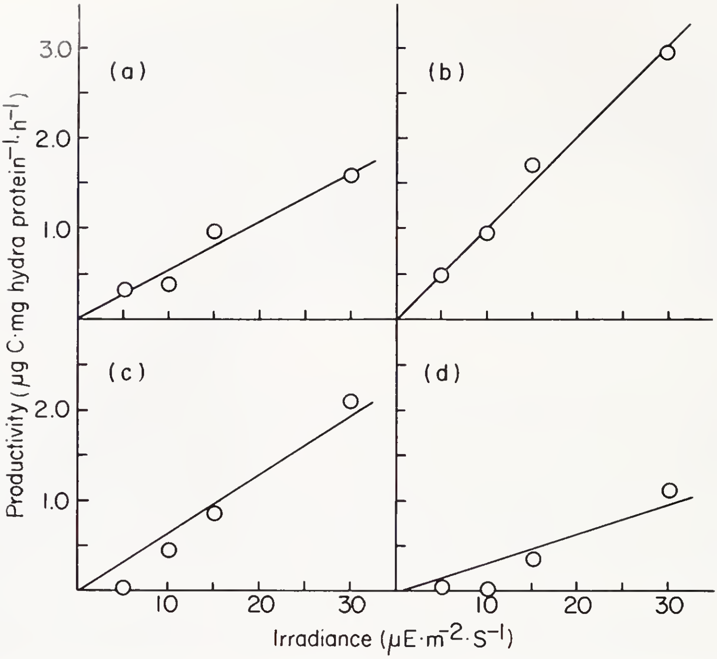

FIGURE 2. Productivity of F/NC hydra at different irradiances. Lines were fit to data points by linear regression. Hydra were maintained at culture irradiances of: (a) 5 $\mu E \cdot m^{-2} \cdot s^{-1}$; (b) 10 $\mu E \cdot m^{-2} \cdot s^{-1}$; (c) 15 $\mu E \cdot m^{-2} \cdot s^{-1}$; (d) 30 $\mu E \cdot m^{-2} \cdot s^{-1}$.

was no significant difference in algal densities of hydra maintained at 15 and 30 $\mu E \cdot m^{-2} \cdot s^{-1}$ (Fig. 5d).

Total chlorophyll per algal cell (Fig. 5c) decreased significantly with increase in culture irradiance (ANOVA $F_{3,15} = 15.98$; $P < 0.001$). Symbionts isolated from hydra

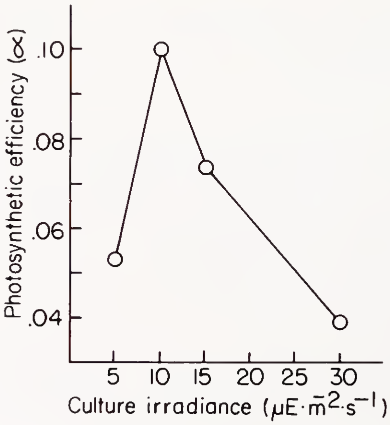

FIGURE 3. Photosynthetic efficiency ($\alpha$) of F/NC hydra as a function of culture irradiance. Units of $\alpha$ are $\mu g\, C \cdot (\mu E \cdot m^{-2} \cdot s^{-1})^{-1} \cdot h^{-1} \cdot mg^{-1}$ protein.

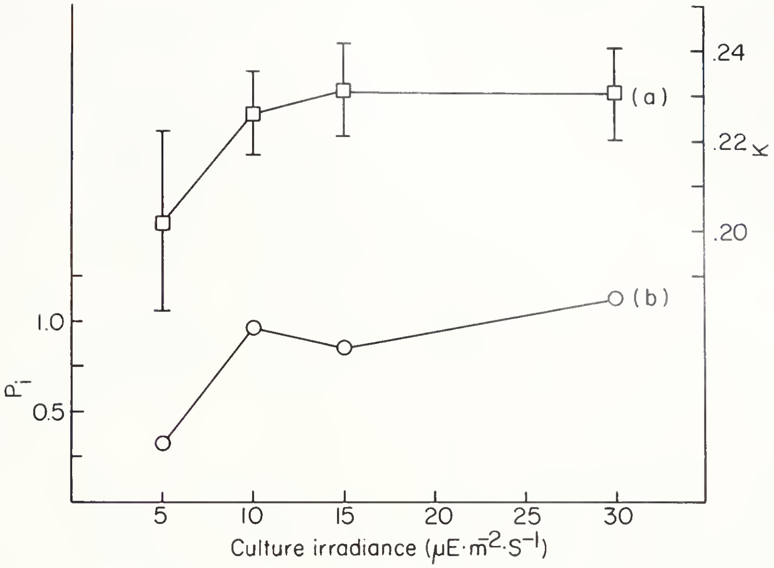

FIGURE 4. (a) Population growth rate constants, k, and (b) productivity, $P_i$, of F/NC hydra at four culture irradiances. Error bars in (a) are ±S.E.. Units for k same as in Table I and units for $P_i$ same as in Figure 2.

maintained at 15 and 30 $\mu E \cdot m^{-2} \cdot s^{-1}$ contained significantly less chlorophyll than those isolated from hydra maintained at the two lower irradiances (Fig. 5c). Data for chlorophyll *a* and chlorophyll *b* gave similar results to those for total chlorophyll, and are not included. There was no significant effect of irradiance on the ratio of chlorophyll *a* to chlorophyll *b* (ANOVA $F_{3,15} = 0.88$; $P = 0.47$), which averaged 1.19 (±0.17; n = 19, ±S.D.). Cell volumes of algae isolated from fed hydra increased with increase in culture irradiance (Table II).

### *Effect of culture irradiance on biomass parameters of 28-day starved hydra*

Biomass parameters of hydra starved for 28 days were compared to initial parameters obtained from the same populations after two days of starvation (Fig. 6). The protein biomass of starved hydra declined to about 10% of initial levels after 28 days of starvation (Fig. 6a).

Algal densities estimated from the number of symbionts per weight of hydra protein in 28-day starved hydra were twice as high as those of 2-day starved hydra (Fig. 6b). Increased numbers of symbionts per digestive cell were also found in 28-day starved hydra (Fig. 6d).

Chlorophyll per algal cell also increased after 28 days of starvation (Fig. 6c). There were proportional changes in chlorophyll *a* and chlorophyll *b*, with the ratio of chlorophyll *a:b* remaining constant at 1.12 for algae isolated from all starved hydra. As was found for symbionts from fed hydra, cell volumes increased with increased irradiance (Table II). However, cell volumes of symbionts from hydra starved for 30 days were greater than corresponding cell volumes of symbionts from fed hydra (Table II).

## DISCUSSION

The nutritional contribution of symbiont productivity to growth of green hydra was evaluated by measuring changes in biomass of hydra maintained under differ-

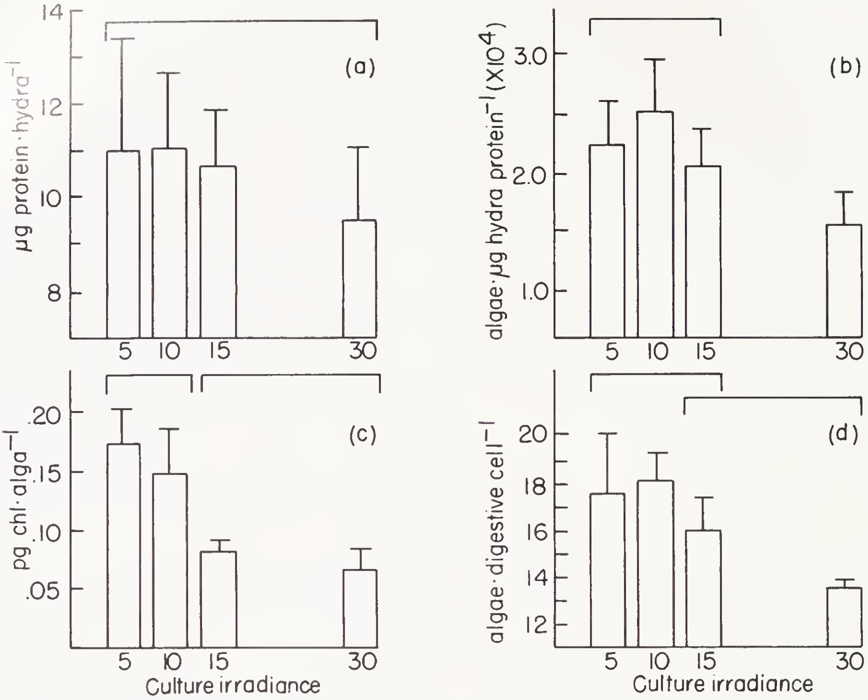

FIGURE 5. Final biomass parameters of F/NC hydra maintained at four irradiances for 4 weeks and fed 4×/week with *Artemia* sp. nauplii. Each bar in 5(a)–5(c) represents the mean of 5 replicate samples of 100 hydra each. Bars in 5(d) are mean values for 3 replicate samples of 100 digestive cells each. Error bars represent standard deviations of the mean. The horizontal brackets connect data sets which were found to be not significantly different at the $P > 0.05$ level.

ent irradiances. The results show that culture irradiances ranging from 5 to 30 $\mu E \cdot m^{-2} \cdot s^{-1}$ had no significant effect on both growth rate of fed green hydra and survival of starved green hydra. While the productivity of fed green hydra increased with an increase in culture irradiance, hydra growth was independent of culture irradiance. These results suggest that the amount of carbon fixed by the symbiotic algae and potentially available to the hydra via translocation or digestion is not transferred

TABLE II

*Cell volumes of symbiotic algae from F/NC hydra either fed or starved for 30 days at four culture irradiances*

| Culture irradiance ($\mu E \cdot m^{-2} \cdot s^{-1}$) | Algal cell volume ($\mu m^3$) | |
|---|---|---|
| | From fed hydra | From starved hydra |
| 5 | 10.4 | 18.5 |
| 10 | 12.1 | 20.6 |
| 15 | 15.6 | 21.5 |
| 30 | 19.0 | 27.2 |

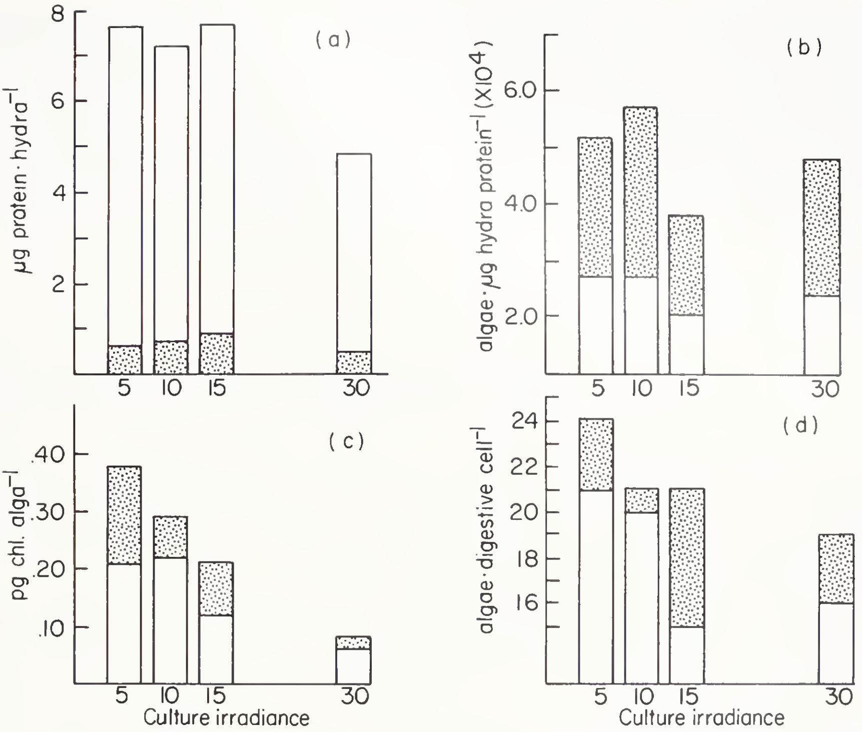

FIGURE 6. The effect of starvation on biomass parameters of F/NC hydra maintained at four irradiances. Initial biomass parameters were measured in 2-day starved hydra (□); final biomass parameters in 28-day starved hydra (▨).

directly into animal biomass of the symbiotic association. Similar results were obtained in a previous study on the symbiotic sea anemone *Aiptasia pulchella,* where changes in sea anemone biomass in fed and starved animals were not related to the productivity of the symbiotic algae at different irradiances (Muller-Parker, 1985).

Hydra were maintained at irradiances below those required for light-saturated photosynthesis by the algae. Symbiotic algae were smaller at lower irradiances, and responded to decreases in irradiance with proportional increases in chl *a* and chl *b*. The greatest increase in productivity occurred between hydra maintained at 5 and 10 $\mu E \cdot m^{-2} \cdot s^{-1}$, and photosynthetic efficiency declined with further increase in culture irradiance.

Algal-animal biomass parameters of green hydra changed in response to feeding and culture irradiance. Algal density in fed hydra changed in response to culture irradiance, as both the number of algae per weight of hydra protein and the number of algae per digestive cell were lower for hydra maintained at the highest irradiance (Fig. 5b, d). In spite of lower algal densities, changes in the ratio of plant to animal biomass were less pronounced, since algal cell volumes increased with increase in culture irradiance (Table II). Algal densities of 28-day starved hydra were twice those of fed hydra (Fig. 6b). Since algal cell volumes from these hydra were much greater than those of algae from fed hydra (Table II), the ratio of plant to animal biomass increased greatly with starvation of the host.

We have measured the response of one hydra-algal symbiosis to different culture irradiances. Whether other hydra-algal associations show similar responses to changes in culture irradiance is presently unknown. Although the symbiotic algae (NC64A) used in this study were characterized by Mews and Smith (1982) as "low maltose releasers," Mews and Smith (1982) found that there was no difference in the growth rates of hydra (English strain) containing "low maltose releasers" and "high maltose releasers." The amount of carbon fixed by symbionts, the amount of carbon translocated (or digested), and the ratio of algal to animal biomass of a given hydra association may variously affect the response of the symbiotic association to light.

### *Population growth of fed hydra and survival of starved hydra maintained at four culture irradiances*

Population growth of fed green hydra was independent of photon flux density between 5 and 30 $\mu E \cdot m^{-2} \cdot s^{-1}$ (Table I). These results support those of Epp and Lytle (1969), who showed that light intensity had no effect on the number of buds produced by the Kenilworth strain of green hydra. Two earlier studies have shown a positive correlation between irradiance and growth rates of symbiotic paramecia (Karakashian, 1963; Pado, 1965), but food was limiting in both experiments. The effect of irradiance on growth rates of symbiotic paramecia was less pronounced in cultures fed bacteria than in bacteria-free cultures (Pado, 1965).

As the productivity of hydra increased with an increase in culture irradiance (Fig. 4b), it appears that growth rates of fed hydra are regulated by factors other than light-dependent carbon fixation. Muscatine and Lenhoff (1965b) showed that the contribution of symbiotic algae to hydra growth was dependent on whether or not food was limiting. When hydra were fed to repletion with brine shrimp, growth rates of green and aposymbiotic hydra were identical. When food was limited, growth rates of green hydra exceeded those of aposymbiotic individuals (Muscatine and Lenhoff, 1965b). Similar results were obtained with symbiotic and aposymbiotic paramecia (Karakashian, 1963). Thus, the similarity in growth rates for the four experimental hydra populations may not be surprising, as equal rations of brine shrimp were provided to all cultures. Although the number of brine shrimp ingested by hydra maintained at the four irradiances was not measured, it is likely that irradiance had no effect on the ability of hydra to capture shrimp since Clayton (1984) found the number of shrimp captured and ingested by green hydra to be the same under both dark and light maintenance conditions.

All other studies on the effect of light on growth of green hydra have dealt with variations in daily photoperiod. No significant difference in budding rate was found for Kenilworth green hydra maintained on either a 12:12 h L:D photoperiod or continuous light (Epp and Lytle, 1969). Douglas and Smith (1984) found that green hydra maintained under continuous light grew more slowly than those maintained on a 12:12 h L:D photoperiod. These studies support the results obtained here, *i.e.,* that there is no positive correlation between total irradiance and growth rates of fed green hydra.

Survival of starved green hydra was not affected by culture irradiance. Although symbiotic hydra starved in the light survive longer than those starved in the dark (Stiven, 1965b; Rahat and Reich, 1980), these results indicate that the total photon flux density does not have a significant impact on the length of time symbiotic hydra survive. It would be interesting to determine the effect of very low irradiances (0–5 $\mu E \cdot m^{-2} \cdot s^{-1}$) on the survival and productivity of starved symbiotic hydra.

The decline in numbers of hydra with days of starvation (Fig. 1) was described by a second order polynomial equation. As some hydra continued to produce buds for up to 20 days of starvation, hydra mortality was somewhat offset by the production of new individuals. Hydra populations did not decline appreciably until the 40th day of starvation. Rahat and Reich (1980) observed buds on green hydra starved for six weeks, and they were able to maintain starved symbiotic hydra in M solution under continuous light for eight months. Unfed hydra cultures were maintained for up to 10 weeks by Douglas and Smith (1984), although survival data were not reported for the starved cultures. Epp and Lytle (1969) showed that there was no significant difference in the number of buds produced by Kenilworth green hydra maintained at two light intensities for 14 days and starved. In their study, bud production stopped after 8 days of starvation in both high and low light cultures. However, the hydra were not pre-acclimated to the high light intensity before the experiment was conducted. Photoperiod had a significant effect on bud production of starved hydra; more buds were produced by Kenilworth green hydra maintained on a 12:12 L:D cycle than by those maintained under continuous light (Epp and Lytle, 1969).

### *Productivity of green hydra*

The maximum rate of carbon fixation, 3 $\mu g\ C \cdot mg$ hydra $protein^{-1} \cdot h^{-1}$, was measured at 30 $\mu E \cdot m^{-2} \cdot s^{-1}$ in hydra acclimated to 10 $\mu E \cdot m^{-2} \cdot s^{-1}$ (Fig. 2b). Normalized to weight of chlorophyll *a*, this rate is 1.40 $mg\ C \cdot mg\ Chl\ a^{-1} \cdot h^{-1}$. As this was a light-limited rate of carbon fixation, it is likely that the productivity of green hydra is at least as high as that of marine symbiotic cnidarians where assimilation numbers range from 1.0 to 3.9 $mg\ C \cdot mg\ Chl\ a^{-1} \cdot h^{-1}$ (Muscatine, 1980).

The productivity of fed green hydra increased with an increase in culture irradiance, with the greatest increase in productivity observed as irradiance increased from 5 to 10 $\mu E \cdot m^{-2} \cdot s^{-1}$ (Fig. 4b). As the productivity of hydra increased from 0.3 to 1.15 $\mu g\ C \cdot mg\ protein^{-1} \cdot h^{-1}$, and neither population growth nor hydra protein biomass were significantly different at the four irradiances, increased carbon fixation by the symbiotic algae did not result in an increase of hydra biomass. However, the curves in Figure 4 suggest that at low irradiances productivity and growth of green hydra may be related. It is not known if photosynthetically fixed carbon was stored in algal lipid or carbohydrate pools, as these were not measured. Other sinks for photosynthetically fixed carbon in symbiotic associations are listed by Muscatine *et al.* (1984). Algal-derived carbon acquired by the animal may be respired or released as particulate or dissolved organic carbon. These parameters were not measured in this study. An energy budget constructed for the symbiotic coral *Pocillopora eydouxi* suggested that about 51% of the photosynthetically fixed energy is used in respiration, 0.9% in growth, and 48% is unaccounted for and probably excreted (Davies, 1984).

Figure 2 shows that light-saturated rates of photosynthesis were not obtained over the range of irradiances measured. Phipps and Pardy (1982) found that light-saturated photosynthesis in green hydra maintained at 60 $\mu E \cdot m^{-2} \cdot s^{-1}$ was obtained at irradiances exceeding 500 $\mu E \cdot m^{-2} \cdot s^{-1}$. In general, photosynthetic efficiency increased with decreasing culture irradiance, suggesting that algae in hydra compensate to culture irradiance. Hydra maintained at 10 $\mu E \cdot m^{-2} \cdot s^{-1}$ were the most efficient at utilizing the available light for carbon fixation (Fig. 3). The low photosynthetic efficiency of hydra maintained at 5 $\mu E \cdot m^{-2} \cdot s^{-1}$ is puzzling, and cannot be explained by any differences in biomass parameters of these hydra (Fig. 5).

### *Effect of culture irradiance on biomass parameters of fed hydra*

Environmental factors can alter the algal-animal biomass ratio of symbiotic hydra. The role of light has previously been addressed by comparing the biomass parameters of hydra maintained in light and in dark. When hydra are transferred from light to continuous darkness the number of algae per digestive cell goes down (Pardy, 1974; McAuley, 1981, 1985b; Steele and Smith, 1981; Douglas and Smith, 1984). When hydra are returned to the light, algal densities return to previous levels (Pardy, 1974; Douglas and Smith, 1984). The effect of irradiance on algal densities in hydra has not been previously examined, although at high light intensity symbiotic hydra lose their algae (Pardy, 1976; Steele and Smith, 1981). In this study the density of symbiotic algae in hydra maintained at four irradiances was estimated with two methods; by calculating the number of algae per $\mu$g hydra protein and by counting the number of algae in digestive cells. Similar results were obtained with both methods. Hydra maintained at the highest irradiance contained fewer algae than those maintained at the three lower irradiances (Figs. 5b and 5d).

Differences in the relative proportion of algal and animal biomass may be less pronounced than suggested by the algal density data, as algal cell volumes in fed hydra maintained at 30 $\mu E \cdot m^{-2} \cdot s^{-1}$ were almost twice those of algae from hydra maintained at 5 $\mu E \cdot m^{-2} \cdot s^{-1}$ (Table II). As cultured symbiotic algae (NC64A) acclimated to different irradiances exhibited a similar reduction in cell volume with decrease in culture irradiance (unpubl. data), these data strongly suggest that culture irradiance, and not some influence of the host, determined the cell size of symbionts *in situ*. Others have found that algae from hydra maintained in the dark were smaller than those from light-maintained hydra (Pardy, 1981; Douglas and Smith, 1984; McAuley, 1985b).

Symbiotic algae adapted to low light with an increase in chlorophyll (Fig. 5c) and a decrease in algal cell volume. The cell volume data in Table II suggests that changes in chlorophyll between algae isolated from hydra maintained at the four irradiances would be even greater if chlorophyll were normalized to cell weight or carbon.

### *Effect of irradiance on biomass parameters of starved hydra*

The protein biomass of F/NC hydra starved for 28 days declined to about 10% of initial levels (Fig. 6a). The final protein biomass of starved hydra was similar in hydra maintained at the four irradiances, but hydra maintained at 30 $\mu E \cdot m^{-2} \cdot s^{-1}$ lost proportionately less protein than the other groups.

Algal densities in hydra starved for 28 days were twice as high as those of fed hydra (Fig. 6b). The difference between initial and final algal densities was less pronounced in the comparison of numbers of algae per digestive cell (Fig. 6d). A similar increase in the number of symbiotic algae per digestive cell in starved hydra was found by Muscatine and Pool (1979), Douglas and Smith (1984), and McAuley (1985a). Douglas and Smith (1984) found that this increase in algal density resulted from a decline in protein content and not from an increase in numbers of algae. Algal cells isolated from starved hydra were larger than those isolated from fed hydra (Table II); similar results were obtained by Douglas and Smith (1984) and by McAuley (1985a). Increase in algal cell size and numbers of algae result in a greater proportion of algal to animal biomass in starved hydra.

This study has attempted to define the role of irradiance in the green hydra symbiosis by comparing growth, productivity, survival to starvation, and algal-animal biomass parameters of F/NC hydra maintained at culture irradiances ranging from 5 to 30 $\mu E \cdot m^{-2} \cdot s^{-1}$. The results indicate that irradiance does not have an effect on the biomass of fed or starved hydra, but that algal-animal biomass parameters are influenced by irradiance and feeding regime.

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# FLOW-ASSISTED MANTLE CAVITY REFILLING IN JETTING SQUID

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## ABSTRACT

During rapid, rearward, repetitive jetting, the mantle of a squid is expanded laterally by hydrodynamic pressure differences between the region of greatest girth and the refilling apertures beneath its downstream margin; this flow-induced expansion assists refilling between jet pulses. Estimates derived from measurements of pressure distributions on a model in wind tunnels indicate that about 50% of the pressure available for refilling is flow-induced at a speed of 3 $\mathrm{m \cdot s^{-1}}$ while 90% is flow-induced at 9 $\mathrm{m \cdot s^{-1}}$.

## INTRODUCTION

Rapid rearward (dorsally directed) locomotion in squid is accomplished by ejection of water from the mantle cavity through a ventrally directed funnel. The cavity then refills through a bilateral pair of one-way valves at the anterior end of the mantle, and the ejection process may be repeated. While clearly less efficient than the swimming of fish (O'Dor, 1982), this jet propulsion system is capable of propelling animals at impressively high speeds at least in brief episodes.

Refilling the mantle cavity demands that the mantle expand its circumference, no ordinary activity for a muscular organ. Two mechanisms have been recognized as contributing to the expansion. Ward and Wainwright (1972) drew attention to a set of very short radial muscle fibers within the mantle, whose contraction could thin the mantle and extend the circumferential muscles. Gosline and Shadwick (1983) showed that, in addition, elastic storage of energy from the expulsion phase played a significant role early in the process of re-expansion.

An additional agency for refilling may be involved, a consequence of the pressure distribution predicted by Bernoulli's principle. If a spherical or ellipsoidal body moves through a fluid, a characteristic distribution of pressures develops along its surface, with the most subambient pressures occurring near or ahead of the region of maximum cross-sectional area normal to flow. If the body is reasonably streamlined (as is a squid), flow follows its surface, moving downstream without substantial separation or reversal, and pressures rise again behind the widest portion. For a jetting squid, maximum girth is adjacent to mantle and mantle cavity; the openings between mantle margin and neck through which refilling occurs are well downstream. Thus pressure inside the mantle cavity should be higher than that outside, creating a net outward force that might augment the actions of radial muscles and elastic connective tissue. [The possibility was mentioned by Ward (1972) but was regarded as inconsequential inasmuch as squid could refill adequately in still water.]

Such flow-induced pressures might reduce the time needed to refill or increase the volume of water in the mantle cavity prior to ejection. Indeed, rapidly moving squid appear to pulse more frequently than those constrained to remain at rest with respect

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to the local water (Gosline, pers. comm.). Functional roles of analogous flow-induced pressures have been previously investigated in fish (DuBois *et al.,* 1974), in an alga (Vogel and Loudon, 1985), and in scallops (Vogel, 1985).

Unfortunately, squid have never been persuaded to perform high speed escape responses with repetitive jetting in any form of confinement. Furthermore, their soft-bodied character, especially when dead, precludes direct measurement of pressures as was done with fish and scallops. Instead, the present study makes recourse to a somewhat abstract physical model—a radially symmetrical body of revolution with a lengthwise row of surface apertures for measurements of the distribution of pressures. Pressures determined on the model are compared to those generated by previously described refilling mechanisms to estimate the contribution of flow-induced pressure differences to the overall process.

## MATERIAL AND METHODS

The model squid (Fig. 1, top) was based on measurements abstracted and averaged from a variety of published and unpublished photographs giving lateral views of *Loligo,* mostly *L. pealei.* Few pictures showed animals in motion, and all reflected full or nearly full mantle cavities. Fins were omitted in the model since they are apparently folded flat against the mantle during rapid jetting (Bradbury and Aldrich, 1969; William Kier, pers. comm.). The extent to which the ventral margin of the mantle hooded the head and the smoothness of fairing of mantle and head varied among the photographs; the model assumes a small head, poor fairing, and little hooding—in short, a worst case from the point of view of smooth flow. [It should be noted that Bradbury and Aldrich (1969) showed an unusually conspicuous gap, nearly that of the present model, between mantle margin and head during refilling in *Illex illecebrosus.*] At the same time, the model permitted "improvement" through the addition of modelling clay.

The model, of aluminum, was 293 mm in length and 50 mm in maximum diameter. Squid are nearly circular in cross-section; the use of a radially symmetrical model is thus only mildly abnormal. Radial symmetry permitted simple fabrication on a lathe and radically reduced the number of measurements needed to map pressure distributions. Thus a single lengthwise row of 24 holes sufficed; these were 10 mm apart in lateral projection, beginning 10 mm behind the upstream (dorsal) end. Each was normal to the surface, 1 mm in diameter, and penetrated to an axial hole running downstream to an exit within the "arms." An additional hole entered the model between head and mantle margin. For pressure measurements all but one of these apertures were occluded by small pieces of plastic adhesive tape. The model was supported by a cylindrical tube that continued the axial hole downstream and by a cylindrical support beneath the widest part of the mantle, diametrically opposite the row of holes.

Pressure measurements were made in air at speeds of 8.75, 16.5, 37.3, and 76.7 $m \cdot s^{-1}$, the lower speeds in the large open circuit tunnel of the Department of Zoology (Duke University) and the higher speeds in the closed circuit tunnel of the School of Engineering. All were differential measurements referred to a static aperture, 1 mm in diameter, 0.1 m downstream from the leading edge of a flat plate parallel to flow that was mounted adjacent to the model.

Where neither compressibility of fluids nor fluid-fluid interfaces complicate matters, the choice of fluid and size of model are quite arbitrary as long as the natural value of the Reynolds number is maintained. Equality of Reynolds number (Re) between reality and model assures dynamic similarity of flows and thus equality of the dimensionless force and pressure coefficients, as discussed by Vogel (1981), Vogel and Loudon (1985), and standard texts in fluid mechanics. Shifting from 20°C seawater to air entails a speed increase factor of 14.5; the use of a model 1.5 times larger than an assumed size of animal mandates a speed decrease of this latter factor. Thus the present speeds in air are functionally equivalent to speeds of 0.91, 1.6, 3.9, and 7.9 $m \cdot s^{-1}$ in seawater providing that pressures are expressed as pressure coefficients. (A pressure coefficient is simply the measured pressure difference divided by the dynamic pressure, the latter being the product of half the density of the medium and the square of the velocity. In practice, pressures were coverted to the coefficients using the density of air and the tunnel velocities; the coefficients were reconverted as needed to equivalent pressures in seawater by substituting the density of seawater and the corresponding speeds in water.)

Pressure differences were measured with a barocell electric manometer and capacitative differential pressure sensor (Datametrics-Dresser, 1174-B1A-5A1-A1 and 570D-10T-2A1-V1X). The combination had a maximum capacity of 1300 Pa and an accuracy, according to the manufacturer, of better than 0.13 Pa (in practice better than any locally available standard). This accuracy corresponds to a pressure coefficient of less than 0.003 for all present measurements. Tunnel speeds were determined with a Pitot-static tube, 3.2 mm in diameter (United Sensor, Inc., PCC 12-KL) and the same barocell and sensor except for the highest speed, for which a simple water manometer (readable to 0.2 mm) provided the necessarily wider range.

Pressure drag (total drag less skin friction) can be viewed as a summation of the streamwise-directed components of the pressures on the surface of a body, where each datum for pressure has been multiplied by the area of surface to which it applies. If skin friction is negligible, it is thus a simple matter to derive values for drag from the pressure measurements; these drag data can be converted to drag coefficients in a manner analogous to the conversion of pressures to pressure coefficients (Vogel, 1981). Thus a general test of procedure and apparatus was carried out by determining the pressure distribution around circular cylinders and from the latter computing drag coefficients—values of 1.0 were obtained at Reynolds numbers of 8000 and 32,000; a commonly cited value is 1.1 (Hoerner, 1965), of which about 3% is skin friction. Similarly, a sphere at Re = 28,000 gave a drag coefficient of 0.42; the accepted value is 0.47.

## RESULTS

### *Pressure coefficients*

Figure 1 (bottom) gives pressure coefficients as a function of position for the model squid. Several points are noteworthy.

(1) These curves are typical of results obtained for streamlined objects (see Discussion). They vary little with changes in Reynolds number (proportional to speed) although the minimum pressure coefficients are slightly more negative at higher Reynolds numbers. Thus the model is well-streamlined—if the decrease in girth is appropriately gentle as flow moves downstream from the point of maximum width then little separation of flow occurs; irregularities further downstream (unfaired mantle margin, head, and arms) are of little consequence. In fact, building up the head area with modeling clay had so little effect that no systematic exploration was made.

(2) The minimum pressure coefficients occur well downstream, not inevitably the case for streamlined bodies of revolution. Clearly location of the pressure minimum near the point of maximum girth will be advantageous for flow-induced refilling of the mantle cavity.

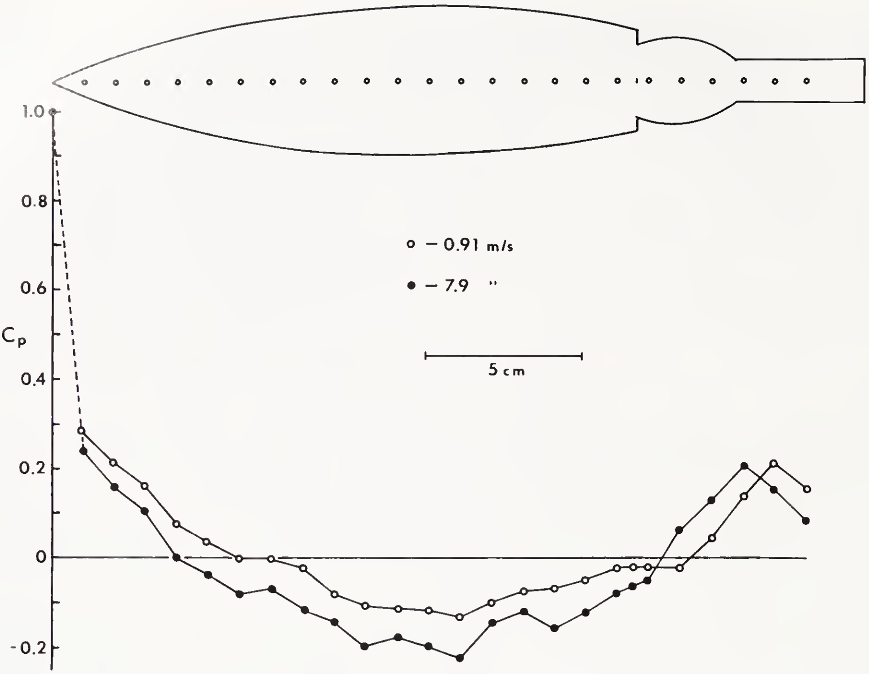

FIGURE 1. Top: the model squid, with flow from left to right, showing location of the apertures for measurement of pressure. Bottom: pressure coefficient ($C_p$) as a function of relative distance downstream from the dorsal extremity; the abscissa is scaled to correspond to the drawing above. Extrapolation to a coefficient of unity at the upstream extremity (dotted line) presumes the existence of a normal forward stagnation point. Speeds refer to equivalent values in seawater; all data for the two intermediate speeds fall between those shown for the maximum and minimum speeds.

(3) The pressure coeffients for the mid-mantle region are about −0.15 while those for the region around the head from which refilling must take place are about +0.10; a difference of approximately 0.25 is thus available for refilling the mantle cavity. The corresponding pressure differences in seawater for the four speeds tested are 106, 330, 1950, and 8000 Pa. (The wide range of these latter figures reflects multiplication by the squares of the velocities to obtain pressures from the pressure coefficients.)

### *Pressure drag*

While not directly germane to the present hypothesis, values for pressure drag emerge from these measurements of pressure. Expressed as drag coefficients for the four speeds tested, the latter are (in ascending order of speed) 0.070, 0.060, 0.027, and 0.033, all referred to frontal (maximal cross-sectional) area. Dividing these values by 13.6 (the ratio of total surface to frontal area) shifts the reference to total surface or "wetted" area. Dividing the values instead by 2.13 (the ratio of volume to the two-thirds power, a nominal area, to frontal area) shifts the reference to $V^{2/3}$.

These coefficients are about an order of magnitude below those of cylinders and spheres, cited earlier. There is no obvious discontinuity in the data corresponding to the drop in pressure drag attending the delay of separation as the boundary layer becomes turbulent (Vogel, 1981). Both these features of the data indicate good streamlining with little separation and low total drag for the model. If this crude model gives such a good account of itself then it is likely that real squid moving rearward are well-streamlined.

## DISCUSSION

Evaluation of the contribution of flow-induced pressure differences to mantle cavity refilling requires two further items of information: (1) the pressure available for refilling in the absence of any direct hydrodynamic effect and (2) the speeds at which squid move during rearward repetitive jetting.

Three separate approaches to estimating the non-hydrodynamic pressure available are currently practical. First, a very rough upper limit is suggested by the capability of the radial muscle of the mantle. The latter underlies about 10% of the mantle surface (Bone *et al.*, 1981); such muscle should be capable of a stress output of 100 $kN \cdot m^{-2}$ in the non-isometric contraction by which the mantle wall is thinned, or 10 $kN \cdot m^{-2}$ referred to mantle area instead of muscle cross-section. Half of the output will be lost since the mantle cannot expand longitudinally, leaving 5 $kN \cdot m^{-2}$ or 5000 Pa. Indeed, this figure may considerably exaggerate what the muscle can do in the latter part of mantle expansion when it is already substantially contracted. And it is in this hyperexpansion phase that the elastic recoil with which mantle expansion begins is no longer significant (Gosline and Shadwick, 1983). Conversely, at near maximum expansion, flow-induced pressures should be greatest.

Second, from observed rates of refilling in non-swimming squid together with the size of the openings through which refilling occurs, Trueman (1980) calculated an inhalent pressure of 1200 Pa. And third, rough but direct measurements of peak refilling pressures are given by Trueman (1980) as 1500 Pa and by Gosline and Shadwick (1983) as 1000 Pa.

More problematical are the speeds attained by swimming squid during the rearward bursts of repetitive jetting. The animals are notoriously ill-adapted to survive in any non-pelagic situation (Summers and McMahon, 1974); and high-speed, repetitive jetting seems never to have been elicited under controlled and reproducible circumstances. Johnson *et al.* (1972) and Trueman (1975) calculated a speed of 3.5 $m \cdot s^{-1}$; but the value is based on the drag coefficient of a sphere (0.47), certainly much too high for a well-streamlined form. Replacing this assumption with a coefficient an order of magnitude lower (the coefficient of pressure drag for the model at the highest speed tested here plus an estimate of skin friction from data cited by Hoerner, 1965) increases their value $10^{0.5}$ times, to 11 $m \cdot s^{-1}$.

Beyond that, the evidence is mainly anecdotal. Alexander (1977) notes without disparagement claims of swimming speeds up to 8 $m \cdot s^{-1}$. Lane (1960) mentions reports of squid leaving the water vertically and clearing deck railings 4.5 to 6 m above a calm sea. Assuming gravitational deceleration only, these heights imply exit speeds of 9.5 to 11 $m \cdot s^{-1}$. Arata (1954) notes an airborne trajectory about 2 m in height and 14 m horizontally; ignoring drag and assuming a normal ballistic trajectory gives an exit speed of about 12 $m \cdot s^{-1}$. Both of these simple calculations, though, may give overestimates: not only do they ignore drag and lift (minor matters for a squid in air) but they neglect the possibility that the last jet of water may be ejected while the squid is in air. And the latter certainly occurs—the photographs of Cole and Gilbert (1970) show that acceleration can continue while an animal is airborne.

In short, it seems reasonably certain that a squid can generate refilling pressures of around 1200 Pa. Similarly, it appears that a squid, pursued by a predatory fish, can achieve speeds in the range of 5 to 10 $m \cdot s^{-1}$. The present study indicates that the net pressure coefficient available for flow-induced refilling is about 0.25. Figure 2 uses

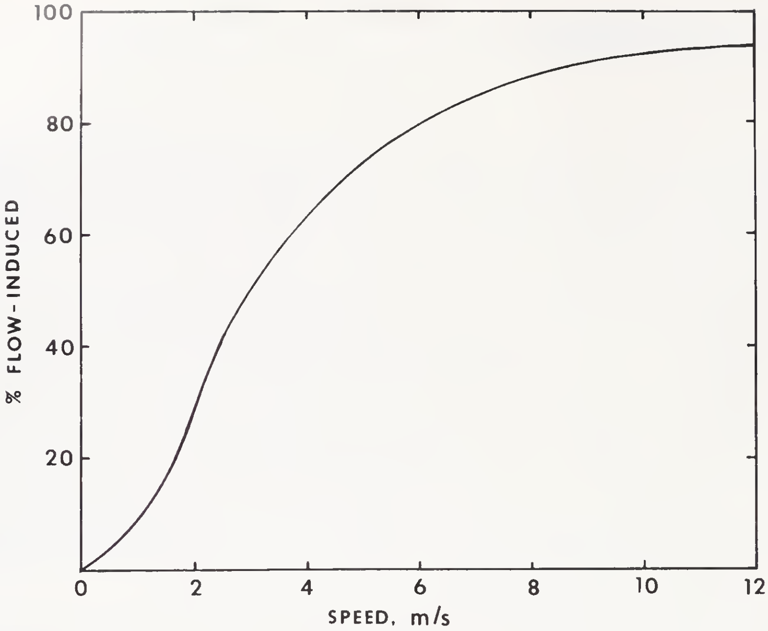

FIGURE 2. The relative contribution of flow-induced pressure differences to total pressure available for refilling as a function of rearward swimming speed, based on present data.

this datum to estimate the relative contribution of hydrodynamics to refilling as a function of swimming speed.

It appears that flow-induced pressure accounts for between 50 and 90% of the total pressure available for refilling in squid during rapid repetitive jetting. Thus the phenomenon is a substantial one. It should permit both more rapid refilling and a greater overall inflation of the mantle cavity and hence a greater maximum swimming speed. It has the virtue of being increasingly effective as speed increases and will be at its best near full inflation, where elastic recoil is minimal and radial muscles have already shortened considerably. It is unlikely, though, that a squid can save energy through the use of flow-induced pressures. Any force that pulls the mantle outward and contributes to refilling is a force against which the circular muscles will have to contend during the expulsion phase, and the distances of mantle movement outward and inward must be the same. But inasmuch as repetitive rearward jetting constitutes an escape response, speed must be far more relevant than any energetic considerations.

The present model is certainly an imperfect squid, although just how imperfect is uncertain in the absense of information on the shape of a squid during repetitive high-speed jetting. Examination of the available data on pressure distribution around streamlined bodies of revolution, though, suggests that a detailed investigation of the effects of taper, size of gap between mantle margin and head, and head size is not warranted. Graphs of pressure coefficients *versus* location simply don't vary much with either the details of shape or, over a very wide range, the Reynolds number. (The present data span a range of Reynolds numbers from 155,000 to 1,350,000.) Arnstein and Klemperer (1936) give a curve similar to the present data for a full-size airship for which the Reynolds number, based on length, was over $10^8$. Goldstein (1938)

(Fig. 215a) presents data at Re = $3 \times 10^6$ especially similar to the present results. Data for a model of the alga, *Halosaccion,* at Re = 14,000 (Vogel and Loudon, 1985), are similar as well, as are data obtained using a toy water rocket (Park Plastics Co., Linden, New Jersey) whose fins had been removed at Re = $10^5$. Minimum pressure coefficients range only from −0.15 to −0.2. The main consistant variation is that the location of the minimum is further downstream for shapes with less blunt upstream ends. Thus the indifference of the present model to alterations in shape comes as little surprise. And, while a squid of about 0.2 m in length was assumed in calculating equivalent swimming speeds in water, the pressure coefficients vary so little with Reynolds number that the data should apply about as well over a considerable size range.

Other, more serious limitations of the present study ought to be borne in mind. Measurements were carried out in steady flow, whereas jetting is an unsteady motion (*sensu* Daniel, 1984). And the act of refilling will to some unknown extent relieve the predicted pressure differences. The major uncertainty, though, comes from the lack of data on actual swimming speeds; since flow-induced pressure is approximately proportional to the square of speed, mere refinement of model and experimental conditions would be of limited usefulness in the absence of better information on swimming speeds in nature.

Nevertheless it is difficult to imagine how substantial flow-induced pressures can be avoided. Indeed, at least one other structural feature of squid is consistent with the occurrence of such pressures during rapid, rearward jetting. The pen more nearly encircles the mantle near the upstream (dorsal) end, where flow will generate compressive forces (positive pressure coefficients in Fig. 1), and thus perhaps provides a measure of compression resistance in a manner analogous to the skull of a fish.

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# METAL REGULATION AND MOLTING IN THE BLUE CRAB, *CALLINECTES SAPIDUS:* COPPER, ZINC, AND METALLOTHIONEIN

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## ABSTRACT

Blue crab, *Callinectes sapidus,* hemolymph and digestive glands were examined at different stages of the molt cycle to determine whether molting affected tissue and cytosolic partitioning of copper and zinc and, if so, whether metallothionein was involved. The crabs used in these determinations were not exposed to elevated concentrations of copper or zinc. Concentrations of hemocyanin, copper, and zinc in the hemolymph all decreased significantly during molt. They were lowest at the soft crab stage ($A_2$) and highest during premolt ($D_1$–$D_3$) and intermolt ($C_4$). The digestive gland copper concentrations also were highest during premolt ($D_1$–$D_3$) and lowest in the papershell stage ($B_1$). Zinc followed the same general pattern in both hemolymph and digestive glands. The cytosolic distributions of copper and zinc were determined in the digestive glands using gel filtration chromotography. The elution profiles showed that the percentages of copper and zinc on metallothionein ranged from 10% copper/90% zinc at $D_1$ to 100% copper at $B_1$. The estimated concentrations of metallothionein were highest during intermolt ($C_1$) and premolt ($D_1$–$D_3$) and lowest during the papershell ($B_1$). The observed changes in the tissue and cytosolic partitioning of copper and zinc are consistent with the physiological changes occurring in the crabs. These observations support the hypothesis that metallothioneins are naturally occurring proteins that are actively involved in the synthesis of hemocyanin and zinc regulation during the normal processes of growth in blue crabs.

## INTRODUCTION

Molting in crustaceans and the biochemical mechanisms that control the turnover and synthesis of metallothioneins are complex and possibly interacting physiological and biochemical processes. Before this investigation, no attempt had been made to link these two physiological activities. This effort, therefore, was designed to determine if changes in the metal composition and amount of metallothionein in the blue crab, *Callinectes sapidus,* are correlated with the molt cycle.

Molting has been examined in detail in a variety of marine and freshwater crustaceans. Skinner (1985) provides an overview of the complex physiological and biochemical processes that are involved in crustacean molting and regeneration with an emphasis on hormonal control. Recently, investigators demonstrated that there are changes in hemocyanin concentrations in the hemolymph of lobsters and blue crabs (Hagerman, 1983; Mangum *et al.,* 1985) during the molt cycle and also osmoregulatory and biochemical changes in molting blue crabs (Towle and Mangum, 1985; Wheatly, 1985). Additionally, there are some reports of changes in metal metabolism

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during molt (Martin, 1975; Adams *et al.,* 1982) which may involve the metal-binding protein metallothionein.

Metallothionein has been characterized as a detoxifying protein that is synthesized in response to elevated metal concentrations both internally and in the environment. It is a low molecular weight protein (<10,000 $M_r$) which binds cadmium, copper, zinc, and mercury, and contains high concentrations of cysteine (>20%) (Cherian and Goyer, 1978). Since initial isolation by Margoshes and Vallee (1957), metallothioneins have been studied extensively in mammals and invertebrates (Kojima and Kagi, 1978; Nordberg and Kojima, 1979; Roesijadi, 1981). These investigations have been concerned primarily with the involvement of metallothionein in detoxification of metals in animals and with the characterization of the protein molecule. Currently there is renewed interest in determining "normal" function of metallothioneins and the metabolic processes that control constitutive levels of metallothioneins in unstressed organisms (Engel and Brouwer, 1984a; Cousins, 1985; George and Viarengo, 1985; Engel and Roesijadi, in press).

Metallothioneins have been demonstrated in marine crustaceans exposed to elevated levels of cadmium and copper in their water or food (Olafson *et al.,* 1979a, b; Overnell and Trewhella, 1979; Rainbow and Scott, 1979; Overnell, 1982; Wiedow *et al.,* 1982; Engel and Brouwer, 1984b; Engel *et al.,* 1985; Engel and Brouwer, 1986; Overnell, 1986). In the blue crab, metallothioneins have been shown to bind cadmium, copper, and zinc in both laboratory exposed animals (Brouwer *et al.,* 1984; Engel *et al.,* 1985) and in animals from contaminated environments (Weidow *et al.,* 1982; Engel and Brouwer, 1984b). Gel chromatography of the digestive gland cytosol yields proteins that have an estimated molecular weight of 7,000–10,000 $M_r$ and bind the three metals. Further purification of these proteins by ion exchange chromatography demonstrated that there was one protein that binds cadmium and zinc and another that binds copper. Further characterization of the cadmium metallothionein showed that it had the characteristics of the mammalian metallothionein (Brouwer *et al.,* 1984). In our investigations with blue crab metallothioneins, we have observed apparent correlations between the physiological condition of the crab and the metal distribution in the cytosol.

The present research effort was designed to investigate whether molting contributes to variability in metallothionein concentration and metal composition in blue crabs. These experiments were designed to examine total copper and zinc in the hemolymph and digestive glands and the cytosolic distributions of copper and zinc in the digestive glands of crabs during six different stages of the molt cycle.

## MATERIALS AND METHODS

### *Animals*

All blue crabs used in these experiments were collected from estuarine waters in the vicinity of Beaufort, North Carolina. Three groups of crabs were obtained throughout the summer of 1985 and these animals included premolt, soft, papershell, and intermolt crabs, and were comprised of about 75% immature females. The temperature and salinity at the time of collection was 20 to 22°C and 31 to 33‰.

The designations of molt stage were according to those described by Mangum (1985) for the blue crab and by Passano (1960) for brachyuran molt stages.

### *Tissue metal measurements*

The concentrations of copper and zinc were determined in samples of digestive gland and hemolymph from individual blue crabs. Hemolymph samples were collected by severing the fifth pereiopod at the meropodite and collecting the fluid in a polyethylene vial. A portion of the hemolymph was taken for metal analysis and the remainder was used for determination of hemocyanin concentration. The crabs were killed by removing the carapace, and the digestive gland was dissected and used for total metal measurements and cytosolic metal determinations. The tissue used for determination of cytosolic distribution of metals was flash-frozen and stored in a freezer at −70°C.

Tissue samples used for metal analysis were oven dried at 100°C for 48 hours and wet ashed with concentrated $HNO_3$ at 90°C. Residue was dissolved in 0.25 *N* HCl, and concentrations of copper and zinc were measured using flame atomic absorption spectrophotometry. Preparative and measurement techniques were calibrated against the National Bureau of Standards Oyster Reference Material #1566.

Measurements of hemocyanin concentrations in the blue crab hemolymph samples were made by Dr. Marius Brouwer (Duke University Marine Laboratory/Biomedical Center, Beaufort, North Carolina). The clotted hemolymph was homogenized, centrifuged, and hemocyanin concentration was determined spectrophotometrically using the extinction coefficient of the native undissociated protein (Johnson *et al.,* 1984).

## *Chromatography*

Individual crab digestive glands were used in each chromatographic separation. Frozen tissue samples were weighed and then placed in 10 ml of 60 m*M* Tris buffer, pH 7.9, with 2 m*M* 2-mercaptoethanol in an ice bath and homogenized 30 seconds with Brinkman Polytron[1] homogenizer. The homogenate was then centrifuged at 105,000 × *g* for 90 minutes at 4°C. The resulting supernate was considered to be the cytosolic fraction. Mass balances were conducted on each sample, and aliquots of the homogenate and cytosol and the entire pellet with fat cap were analyzed for metals according to the procedure outlined above.

Gel-chromatography of the cytosolic fraction was done on a Sephadex G-75 column (2.6 cm × 66 cm). The elution buffer was 60 m*M* Tris, pH 7.9, with 2 m*M* 2-mercaptoethanol. Individual 7-ml fractions were analyzed for absorbance at 250 nm, and for concentrations of copper and zinc were measured directly by flame atomic absorption spectrophotometry. The column was standardized with blue dextran ($>10^6$ $M_r$), bovine serum albumin (67,000 $M_r$), ovalbumin (43,000 $M_r$), chymotrypsinogen A (25,000 $M_r$), ribonuclease (13,700 $M_r$), and potassium. The determination of molecular weight was calculated from:

$$K_{av} = \frac{V_e - V_0}{V_t - V_0}$$

The elution characteristics of the column were: $V_e$—the elution volume of an unknown or standard, $V_o$—the elution volume of the high molecular weight material excluded from the column, and $V_t$—the total elution volume of the ionic component or "salt fraction" as measured by potassium. The peak which eluted with an apparent molecular weight of 7,000–10,000 $M_r$, was considered to be the metallothionein fraction.

Further analysis of the metallothionein peak was performed on a DEAE Sephacel ion-exchange column (1.6 × 10 cm). A gradient was generated from 250 ml, 60 m*M*

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[1] Mention of a trade name does not constitute an endorsement by the National Marine Fisheries Service, NOAA.

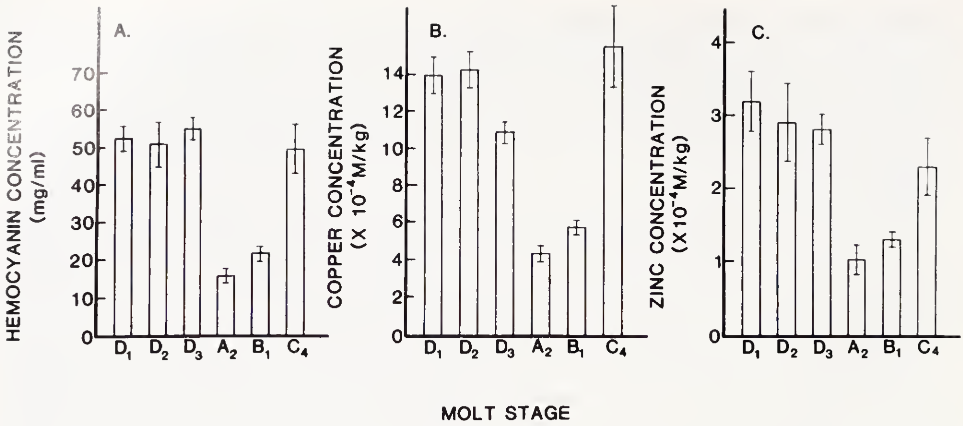

FIGURE 1. Hemolymph concentrations of hemocyanin (A), copper (B), and zinc (C), from blue crabs during different stages of the molt cycle. Molt cycle stages were: premolt white sign ($D_1$), pink sign ($D_2$), and red sign ($D_3$); soft shell ($A_2$); paper shell ($B_1$); and intermolt ($C_4$). Hemocyanin measurements made by Dr. M. Brouwer, Duke Marine Biomedical Center, Beaufort, NC. Each mean represents five or six individual hemolymph samples ± 1 standard error.

Tris, pH 7.9, and 250 ml, 300 m*M* Tris, pH 7.9, both contained 2 m*M* 2-mercaptoethanol. Fractions were analyzed for conductivity and concentrations of copper and zinc.

## RESULTS

### *Hemolymph and tissue metal concentrations*

Blue crab hemolymph concentrations of hemocyanin, copper, and zinc changed during the molt cycle. Hemocyanin concentrations during the soft crab ($A_2$) and papershell ($B_1$) stages were significantly different ($P < 0.05$) from the three premolt stages ($D_1$, $D_2$, $D_3$) (Fig. 1A). The concentrations of copper and zinc followed the same pattern as the hemocyanin and also were different from the intermolt crabs ($C_4$) (Fig. 1B, C). The correlation between copper and hemocyanin would be expected, since copper is an integral part of the hemocyanin molecule. The positive correlation between changes in hemocyanin concentration and zinc concentrations suggests that the zinc may be bound to hemocyanin (Fig. 1C).

The digestive gland concentrations of copper and zinc were reflective of the physiological changes that occur during the molt cycle in the blue crab. Copper concentrations in the digestive gland showed significant decreases ($P < 0.05$) in both the soft and papershell stages (Fig. 2A), and the papershell stage also was lower than the soft crab stage ($P < 0.05$). The differences were 3.4 and 6.0 times lower than the $D_1$ stage of premolt. The observed decreases in copper in the digestive gland are in close correlation with decreases of copper in the hemolymph (Fig. 1B). This means that copper is being lost or excreted, and not conserved by the digestive gland. In the initial portions of the molt cycle, however, it appears that, as the crabs move from intermolt ($C_4$) to premolt ($D_1$–$D_3$), there is an increase in copper in the digestive gland, which may be associated with changes in hemocyanin synthesis and degradation, though this retention of copper does not persist. Zinc concentrations showed the same type of variation during the intermolt and premolt periods as copper, and the soft and

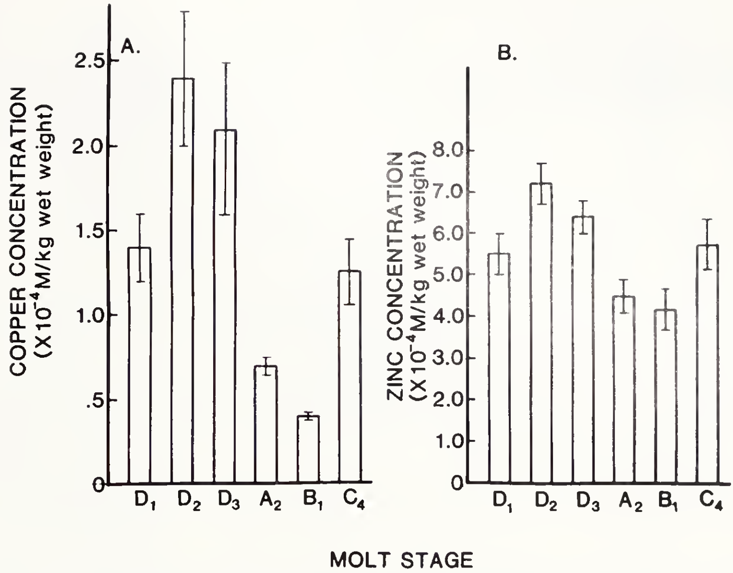

FIGURE 2. Digestive gland concentrations of copper (A) and zinc (B) from blue crabs during different stages of the molt cycle (see Fig. 1). All mean values represent five or six individual samples of digestive gland ± 1 standard error.

papershell stages also were different from premolt crabs ($D_2$), ($P < 0.05$) (Fig. 2B). The concentrations of copper and zinc in the digestive glands of the $C_4$ and $D_1$ stage crabs were in good agreement with concentrations measured previously in intermolt blue crabs collected near Beaufort (Engel and Brouwer, 1984b).

### *Cytosolic metal distribution*

The Sephadex G-75 elution profile for the cytosol prepared from the digestive gland of a $D_1$ stage blue crab showed partitioning of the metals with a pronounced 7–10 K $M_r$ metallothionein-like protein that contained zinc and copper (Fig. 3A). Both zinc and copper also were present in the high molecular weight material (fractions 13–22), but only zinc was present in the very low molecular weight material (fractions 42–52).

Further characterization of the metallothionein-like protein peak (fractions 32–38) was done on a DEAE Sephacel ion-exchange column with a Tris gradient 60–300 m*M* Tris, pH 7.9. Three metal containing peaks were resolved, a zinc component in the breakthrough volume and copper and zinc components that eluted at conductivities of 5.5 and 6.0 mS/cm (Fig. 3B). These conductivities represent Tris concentrations of 110 and 125 m*M* Tris at pH 7.9, which compare favorably to the data of Brouwer and co-workers (Duke University Marine Biomedical Center, Beaufort, NC, pers. comm.) for cadmium metallothionein in blue crabs. Therefore, the protein peak eluting from the G-75 gel filtration column at an apparent molecular weight of 7–10 K $M_r$ will be referred to as metallothionein (MT). The observed separation between the copper and zinc peaks, however, does not necessarily mean that they are separate copper and zinc-binding proteins, but may simply reflect differences in charge characteristics on the protein.

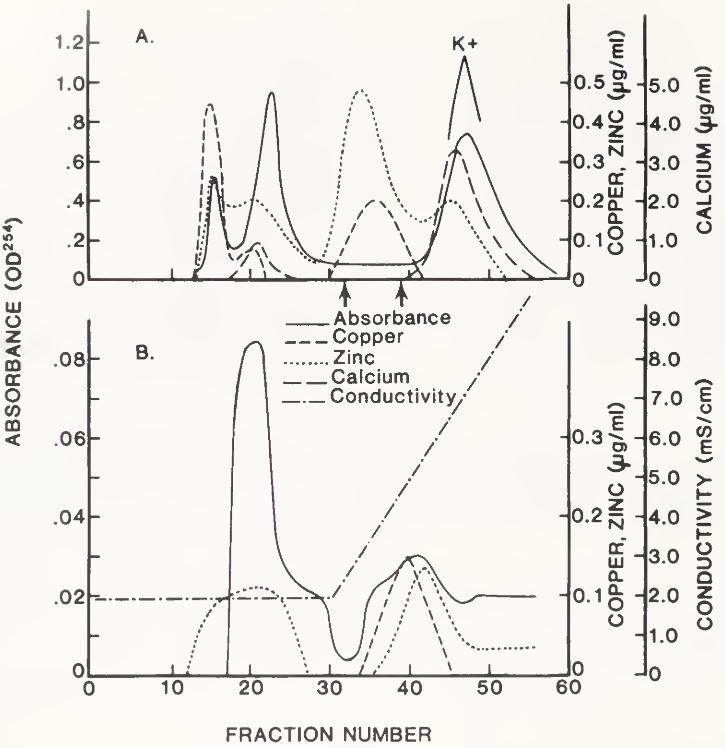

FIGURE 3. Elution profile of the cytosolic fraction of the digestive gland of an intermolt crab showing the partitioning of metals; (A) Sephadex G-75 elution profile column 2.6 × 65 cm with a flow rate of 30 ml/h of 60 m*M* Tris, pH 7.9 with 2 m*M*-2 mercaptoethanol and (B) DEAE—Sephacel ion-exchange column 1.6 × 10 cm using a gradient generate from 250 ml, 60 m*M* Tris, pH 7.9 and 250 ml, 300 m*M* Tris, pH 7.9 and both contained 2 m*M*-2 mercaptethanol. The arrows denote the metallothionein peak that was pooled and subjected to ion exchange chromatography. The peak marked $K^+$ represents the position of the potassium peak (very low molecular weight).

The individual elution profiles of digestive gland cytosol for the six different stages of molting blue crabs show differences in metal distributions that can be correlated with the molting process (Fig. 4). The premolt crabs examined ($D_1$, $D_2$, $D_3$) have a very pronounced metallothionein peak, which is dominated by zinc (Fig. 4A, B, C). Zinc is also present in the high (>60 K, $M_r$), intermediate (~40 K, $M_r$), and very low (<1 K, $M_r$) molecular weight fractions. Copper is present only in the high and metallothionein portions of the profile. In the soft crabs ($A_2$), both zinc and copper are present in the metallothionein region (Fig. 4D), and the relative amount of copper has increased. Otherwise, the distributions of copper and zinc in the elution profiles are the same as before. The metal content of the metallothionein in papershell crabs ($B_1$) is entirely copper with no measureable zinc (Fig. 4E), and the zinc is present only in the high, intermediate, and low molecular weight fractions. Hard shell or intermolt crabs ($C_4$) have a very elevated copper metallothionein with a low amount of zinc present in the same fractions (Fig. 4F), and zinc follow the same pattern as seen in the $A_2$ and $B_1$ crabs.

### *Estimates of total copper/zinc binding proteins*

Calculations to estimate the total metallothionein bound metal in the digestive gland cytosol of the different molt stages were made using the modified relationship of Frazier and George (1983). The relationship is as follows:

$$\text{B.P.} = \frac{[\text{Zn}]}{6} + \frac{[\text{Cu}]}{10} \Bigg/ \begin{matrix}\text{Adjusted}\\ \text{Sample Volume}\\ (10\ \text{ml} = 100\%)\end{matrix} \Bigg/ \begin{matrix}\text{Wet Weight of Tissue}\\ \text{in grams}\end{matrix}$$

The molar concentrations of copper and zinc were calculated from the five peak fractions of each metallothionein peak and were adjusted for applied sample volume and wet weight of tissue. It was assumed that individual metallothionein molecules bind only copper or zinc, and that the blue crab metallothionein binds six zinc atoms [*e.g.*, the *Scylla* protein binds 6 moles of cadmium per mole of protein which is assumed to be the same as zinc (Olafson *et al.*, 1979)], or 10 atoms of copper [*e.g.*, conservative estimate from conversations with M. Brouwer (pers. comm)]. Such calculations are estimations of concentration and are not meant to be quantitative, but do allow for comparisons between groups of crabs.

When the comparisons were made between the different groups of crabs, differences not only were observed in the total amount of protein-bound metal, but also in the relative amount of copper and zinc that was present (Fig. 5). The estimated concentrations of protein- (*i.e.*, metallothionein) bound metal followed roughly the same pattern as total copper and zinc concentrations in tissue (Fig. 5A). When the percentages of metallothionein-bound copper were calculated, they ranged from 7% to 10% of total in the premolt stages, to 35% in the soft crab, to 100% in the paper shell, and 75% at intermolt (Fig. 5B). Such differences show that the cytosolic distributions of metals change during molting and that metal metabolism and metallothionein must be linked directly with the extensive cellular processes that are occurring. This hypothesis is supported further by the observed changes in the percentages of total digestive gland copper and zinc that are present in the cytosolic fraction (Fig. 5C). These calculations, made from the mass balances, show that the percentages of copper in the cytosol at different stages of molting range from 60 to 80 percent. During $A_2$ and $B_1$, when the copper bound to metallothionein is increasing, the percentage of zinc in the cytosol drops from ~75 to 34 percent. This decrease can be accounted for by zinc in the pellet, indicating that there is an intracellular redistribution of metal, which results in some conservation. Since the zinc is in the pellet, it must be incorporated into relatively inert structures or bound to membranes that were not disrupted by the homogenizing process.

## Discussion

The blue crabs used in these experiments were not exposed to artifically elevated concentrations of metals in the laboratory. Therefore, changes in metal concentrations in the hemolymph and the digestive gland and in cytosolic metal distributions were constitutive processes associated with molting. If it is assumed that these changes in metal partitioning are involved in the synthesis of metalloproteins, such as enzymes and respiratory proteins, then a model of metal metabolism and metallothionein function for blue crabs can be developed.

The greatest changes in overall metal concentration and cytosolic distribution of metals occurred at or around the time of ecdysis and early soft crab. At this time,

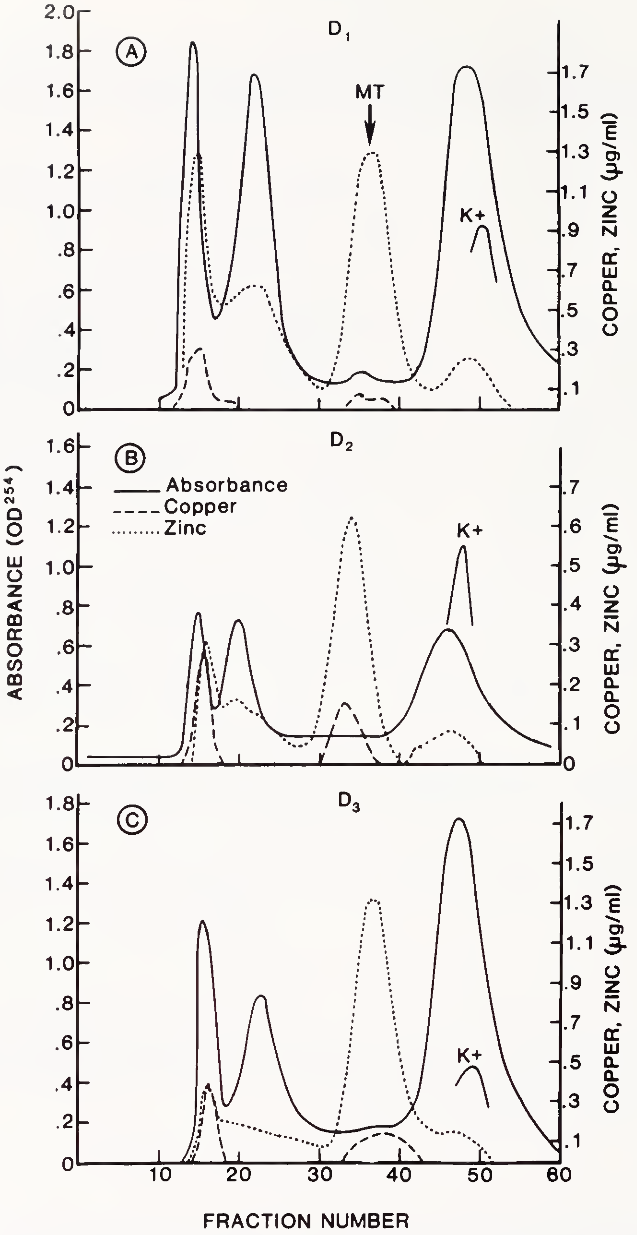

FIGURE 4. A series of representative G-75 elution profiles of digestive gland cytosol from all of the different molt stages that were examined [A($D_1$), B($D_2$), C($D_3$), D($A_2$), E($B_1$), and F($C_4$)]. Conditions were the same as in Figure 3A.

there were significant decreases in the hemolymph concentrations of copper and zinc, which were correlated with decreases in hemocyanin concentrations. These changes in hemocyanin concentration agree favorably with the data of Mangum *et al.* (1985).

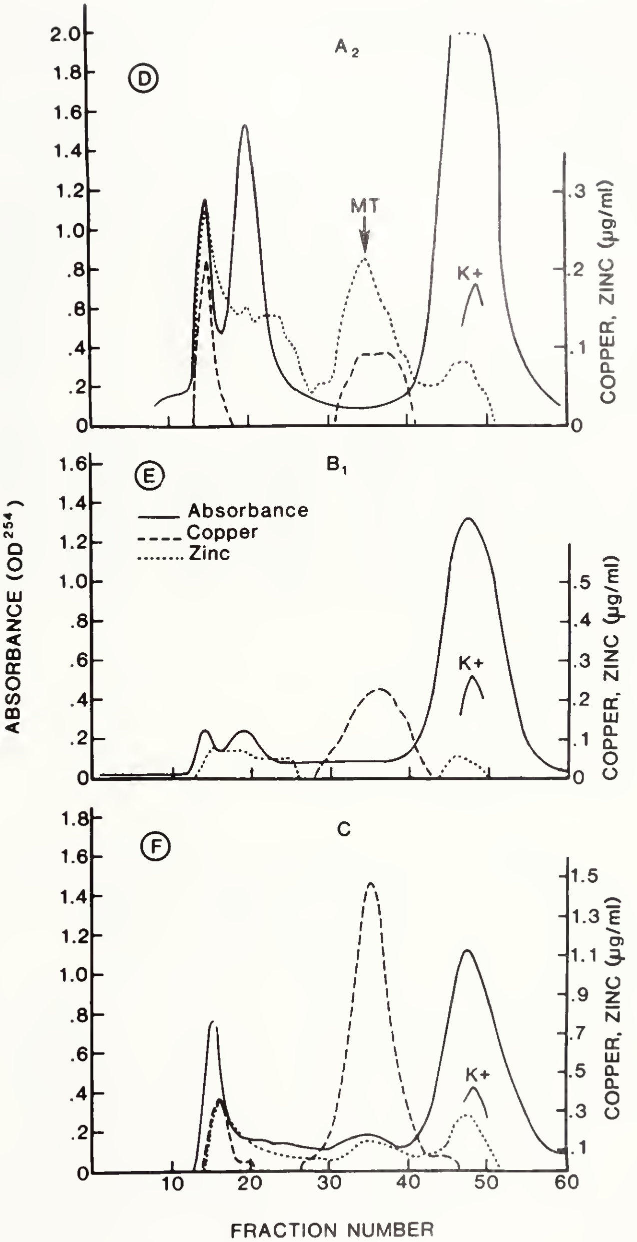

FIGURE 4. (*Continued*)

The decrease in copper is understandable, since hemocyanin is a copper protein, but the associated decrease of zinc strongly suggests that it also is bound to hemocyanin. Zatta (1984) demonstrated with *Carcinus maenas* that each minimal functional subunit of hemocyanin has four zinc-binding sites, and that about 90% of the zinc in the hemolymph was not dialyzable. Brouwer and Engel (1982) also suggested that

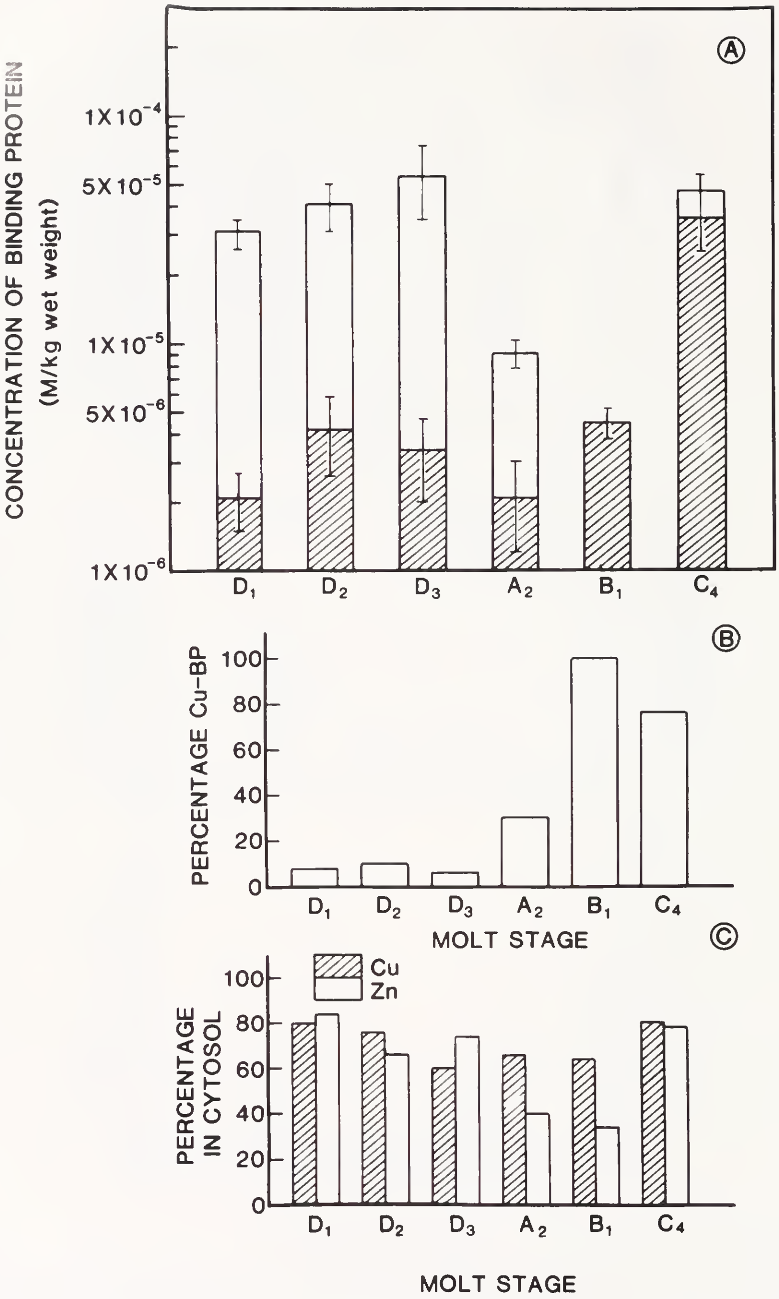

FIGURE 5. Copper and zinc partitioning on the metal-binding protein (*i.e.*, metallothioneins) and in the cytosol of the digestive glands of molting blue crabs. (A) Calculated estimates of total binding protein concentrations (clear bar) and copper-binding protein (cross hatched areas). Means ± 1 standard error (n = 5). (B) Calculated percentage of the total binding protein that binds copper. (C) Percentage of total digestive gland copper and zinc that was present in the cytosol as determined from mass balances on individual digestive glands.

hemocyanin is a major vehicle for the transport of zinc in blue crabs. Additional evidence for the association of zinc with hemocyanin in these experiments comes from the close correlation between the increase in both zinc and hemocyanin in the hemolymph of papershell crabs.

Observed changes in total copper and zinc in the digestive gland during the molt cycle parallel fluctuations in the hemolymph, and the cytosolic distributions of these metals also show large changes. On a molar basis the changes in copper and zinc in the digestive gland between $D_3$ and $B_1$, are quite similar (Fig. 2) and suggest that there is some linkage between the two metals. This linkage also is associated with the changes in the cytosolic distributions of these metals. The most pronounced changes in cytosolic distribution, however, occur between premolt and papershell where the predominant metal bound to metallothionein changes from zinc to copper. These events are relatively rapid and are associated with other physiological and biochemical events.

During this portion of the molt cycle the percentage of copper bound to metallothionein increased as the crabs moved toward papershell (Fig. 5B). The percentage of copper associated with the metallothionein peak changed from 10% copper immediately prior to shedding, to virtually 100% copper at the papershell stage. It appears, therefore, that metallothionein changes from primarily binding zinc to binding copper at the time when the newly molted crabs may be resynthesizing hemocyanin. This correlation supports the hypothesis of Brouwer *et al.* (1986) that copper metallothionein is necessary as $Cu^{+1}$ donor in the synthesis of hemocyanin in blue crabs and lobsters.

Dominance of the copper form of metallothionein continues into the intermolt period and its concentration is high relative to the papershells, but roughly comparable to the total concentrations observed during premolt (Fig. 5A). Even though these animals are stable with regard to molting, the predominance of copper metallothionein suggests a very active turnover of the hemocyanin in the hemolymph of the blue crabs. Senkbeil and Wriston (1981) reported that the T½ for hemocyanin in the lobster was 25.5 days. Since blue crabs may have a higher metabolic rate, their hemocyanin turnover rate may be more rapid. Such turnover rates would require an active pool of $Cu^{+1}$ for degradation, storage, and synthesis of hemocyanin.

In the molt cycle of the blue crab, there are significant decreases in copper in both the hemolymph and digestive gland at the time of molting (Fig. 1B and 2A). Since it appears that the copper is lost by the animal upon molt, portions of the exuvia were measured for copper. These measurements did not show elevated levels of copper in either the molted exoskeleton or stomach and gut linings. Therefore, we do not know the route of copper excretion in the blue crab at molt. Djangmah (1970) indicated that *Crangon vulgaris* excreted copper during molt, and must accumulate it again from either water or food. Such large changes in copper in both hemolymph and the digestive gland also have been demonstrated in the crab, *Cancer irroratus* (Martin, 1975), and in the crayfish, *Austropotamobius pallipes* (Adams *et al.*, 1982). Pathways of metal loss have been examined recently in the shrimp, *Penaeus semisulcatus*, (Al-Mohanna and Nott, in press), using both atomic absorption spectrophotometry and electronmicroscopy with EDAX. They demonstrated that copper and zinc fluctuated during the molt cycle, and that membrane-bound intracellular inclusions were present in cells of the hepatopancreas. These granule-containing cells appear to be sloughed at molt, and the granules which are rich in both copper and sulfur may relate back to metallothionein, and constitute a pathway of excretion for metallothionein bound metals.

While it is possible to relate copper metallothionein to hemocyanin synthesis, it is more difficult to relate zinc metallothionein concentration to any specific synthetic process in the crabs. During the premolt period $D_1$–$D_3$ the predominence of zinc metallothionein is clear, with 90–94% of the total consisting of zinc metallothionein. The decrease in copper metallothionein is probably associated with a decrease in hemocyanin synthesis. Since it is during the premolt period that the new epidermal material is produced, the mobilization of zinc probably correlates with increased need for zinc-dependent enzymes (*i.e.,* carbonic anhydrase) associated with calcification in the new exoskeleton (Henry and Kormanik, 1985). Unlike copper, which varies in concentration in the digestive gland depending upon the environmental conditions, zinc remains remarkably stable (Engel and Brouwer, 1984b). Additional data on tissue concentration of zinc in digestive glands of blue crabs from Long Island Sound to Florida further establish the fact that zinc is highly regulated by blue crabs (D. W. Engel, unpub. data). Also during the molt process, the copper concentration varies by a factor of 6 in the digestive gland, but zinc varies only by a factor of 1.7 (Fig. 2). The absolute changes on a molar basis are similar which suggests a linkage in metabolism.

The data presented here may alter the perception of metallothionein as primarily a detoxifying protein. The function of metallothionein may be one of a regulatory protein for copper and zinc metabolism in unperturbed crabs. Since most of the early research emphasis was on its ability to bind cadmium and mercury, both toxic metals, it is not surprising that the focus was on detoxification. Cousins (1985), however, discusses some of the functions of metallothionein and indicates that it is also involved in stress reactions. Udom and Brady (1980) and Li *et al.* (1980) demonstrated that mammalian zinc metallothionein has the capability of reactivating apo-carbonic anhydrase, and they suggest that metallothionein has a regulatory or transport function in normal physiology. Brouwer *et al.* (1986) has hypothesized that copper metallothionein is the $Cu^{+1}$ donor for the synthesis of crustacean hemocyanin, and the results of this investigation support that hypothesis. They also go a step further in that they show that the pathways of zinc and copper, while linked through their binding to metallothionein, follow different pathways of mobilization and turnover.

The present studies have established that normal physiological processes, such as growth, can influence metallothionein in regard to metal content and concentration in the blue crab. Further, they show that generalized statements with regard to metal metabolism, even within phylogenetic groups, may not be valid, as shown by our data on blue crabs and lobsters. Finally, the present investigation demonstrates the need for basic physiological and biochemical information in order to understand the processes involved in toxicological assessments of environmental impacts of metals.

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# LOSS AND RECOVERY OF LOCOMOTOR BEHAVIOR AFTER CNS LESIONS IN THE SNAIL *MELAMPUS BIDENTATUS*

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## ABSTRACT

To analyze neural pathways underlying the control of locomotion in the snail *Melampus bidentatus,* we lesioned connectives and commissures involved in locomotor control. The results suggest the presence of an independent oscillator in each pedal ganglion which is connected primarily via the pedal commissure. The bilaterally paired cerebropedal and cerebropleural connectives carry redundant information concerned with initiation of locomotion. In the 2–4 weeks after lesions were made, the snails usually regained locomotor coordination.

## INTRODUCTION

Gastropod molluscs are favorable models for investigations of neural control of locomotion because of the relative simplicity of their nervous systems. Several features common to locomotion of many opisthobranch and pulmonate gastropods have been discovered using CNS lesions. These include: (1) command cells in cerebral ganglia that initiate and maintain the locomotor behavior, and (2) pattern generators that organize rhythmic output to muscles. Command centers have been found in cerebral ganglia and pattern generators in the pedal ganglia (Fredman and Jahan-Parwar, 1980, 1983; Jahan-Parwar and Fredman, 1980; Lennard *et al.,* 1980; von der Porten *et al.,* 1982; Getting, 1983; Arshavsky *et al.,* 1985a; Satterlie, 1985).

The use of CNS lesions also revealed the gastropod potential for neural repair and associated recovery of specific behaviors (Price, 1977; Arshavsky *et al.,* 1985b; Moffett and Snyder, 1985). The pulmonate gastropod *Melampus* locomotes by means of a "crawl-step" consisting of several discrete components which make it particularly suitable for behavioral analysis after CNS lesions (Moffett, 1979). The alteration of this behavior following CNS lesions has allowed us to explore the role of particular parts of the nervous system in generation and coordination of locomotion. The sequence and timecourse of recovery from the lesions were also recorded.

## MATERIALS AND METHODS

### *Animals and surgical techniques*

Snails (*Melampus bidentatus*) with shell lengths of 6–10 mm were obtained from Wollaston, Massachusetts, and Poquoson, Virginia. Animal maintenance, anesthesia, and surgical techniques were as described by Moffett and Snyder (1985).

### *Lesions*

The primitively unfused nervous system of *Melampus* contains five paired ganglia and one unpaired visceral ganglion. Five types of lesions were performed: (1) transec-

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tion of the pedal commissure, (2) transection of both cerebral and pedal commissures, (3) bilateral transection of cerebropedal and cerebropleural connectives and statocyst nerve, (4) isolation of the left pedal ganglion by cutting the left cerebropedal and cerebropleural connectives, the statocyst nerve, and the pedal commissure, and (5) excision of the left pedal ganglion (Fig. 1).

### *Behavioral analysis*

The locomotor behavior of each snail was tested 4 to 7 days after surgery and every 2–4 days thereafter. The snail was placed on a glass plate clamped above an angled mirror which allowed us to view and videotape pedal movements. We determined what portion (%) of the foot surface functioned in locomotion by superimposing a grid on the image of the foot. Even when their crawl was abnormal due to lack of coordination or failure of regions of the foot to participate, snails were able to move along the substratum.

Behavioral deficits fell into two categories: (1) failure of musculature in one region of the foot to participate in locomotion (a musculature control deficit), or (2) a deficit in coordination of locomotor movements in different parts of the foot, such as asymmetrical progression of the pedal wave on the right and left sides of the foot. All observations were made by an experimenter who did not know which surgery the snail had undergone.

## RESULTS

### *Cerebropedal and cerebropleural connectives cut*

When cerebropedal and cerebropleural connectives were severed bilaterally (Fig. 1B), snails withdrew into their shells upon recovery from anesthesia. About half did not emerge until they could crawl normally. Although these animals were the slowest of all experimental groups to initiate locomotion after surgery (Fig. 2), they exhibited the fastest recovery of normal pedal waves (Fig. 3). Most deficits in metapodial symmetry in this group involved failure to activate musculature in the posterior third of the foot. They also exhibited abnormal coordination of the propodial region (Fig. 1B).

### *Pedal commissure cut*

All 11 snails which had their pedal commissures cut were crawling by day 7 (Fig. 2). All showed lack of control over the posterior portion of the metapodium on one side (5 animals) or both sides (6 animals), but involvement of the entire foot in locomotion was gradually re-established (Fig. 3). After the entire metapodial musculature became involved in locomotion, the crawl-step was still initiated asymmetrically by contraction of the foot tip on the right or left (Fig. 1C). Once the tip shortened on one side, the pedal wave continued forward either symmetrically or with the initiating side leading. Of 11 snails, 4 regained normal locomotion within 32 days.

### *Pedal and cerebral commissure cut*

When the cerebral commissure was severed in addition to the pedal commissure, side-to-side coordination of pedal locomotion was completely disrupted (Fig. 1D). This surgery delayed the initiation of spontaneous crawling behavior longer than pedal commissurotomy alone (Fig. 2). The time required to re-establish control over

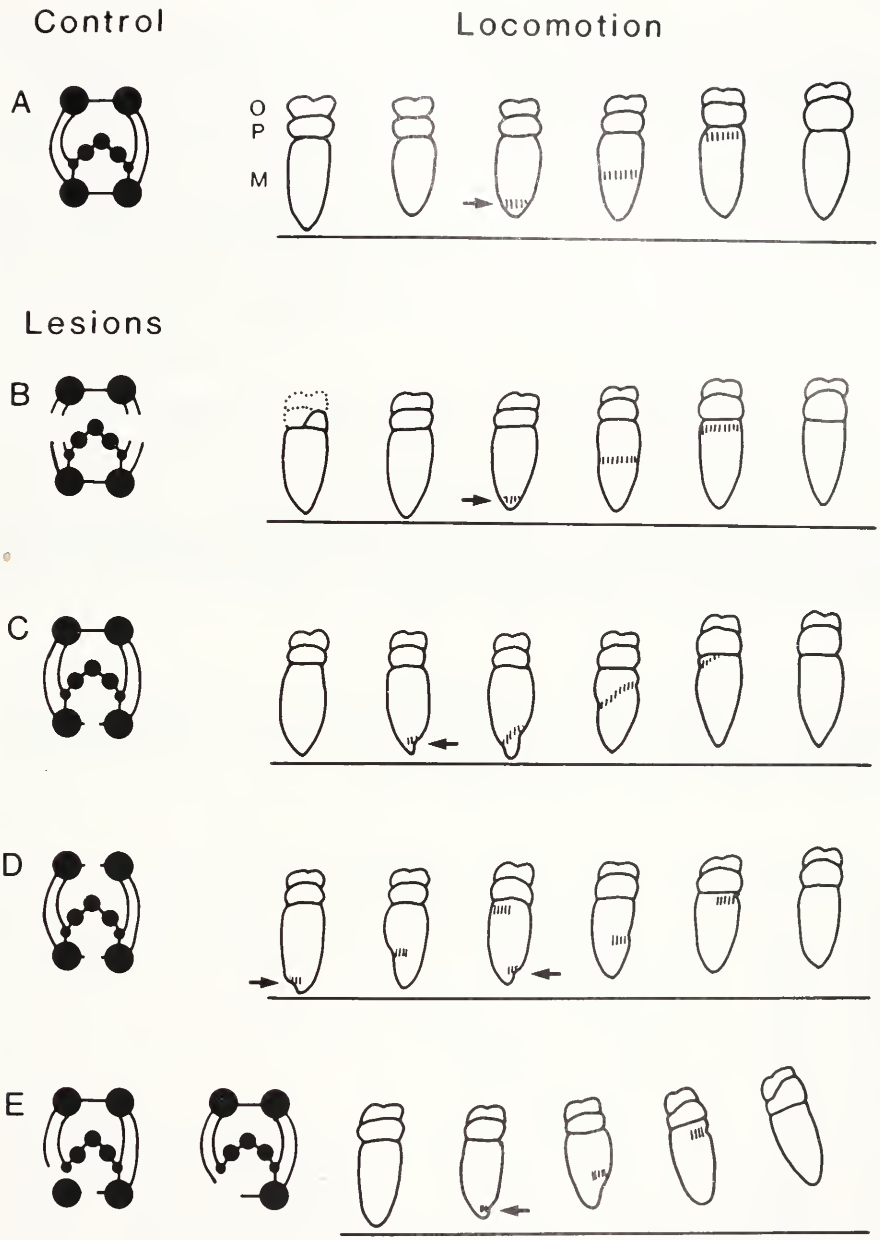

FIGURE 1. Locomotor deficits resulting from CNS lesions. Each line depicts a complete cycle of the crawl-step. Regions on the foot marked by parallel lines are contracting. (A) Control CNS and corresponding locomotion. (O = oral veil, P = propodium, M = metapodium). (B) Right and left cerebropedal and cerebropleural connectives cut. Propodium is often elevated but metapodial wave is normal when snails first locomote. (C) Pedal commissure cut. Wave initiated asymmetrically. (D) Cerebral and pedal commissures cut. Waves on right and left sides independent. (E) Left pedal ganglion isolation and ablation. Each wave pulls animal toward ablated side, requiring continuous course correction. Arrows indicate initiation of pedal wave.

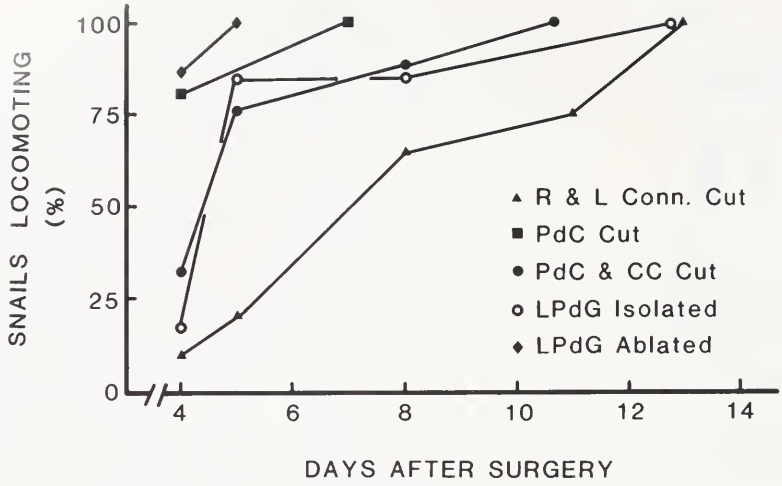

FIGURE 2. Onset of initial attempts at locomotion in groups of snails recovering from each of the five experimental lesions. Triangle = right and left cerebropleural and cerebropedal connectives cut. Square = Pedal commissure cut. Circle (filled) = pedal and cerebral commissures cut. Circle (open) = left pedal ganglion isolated. Diamond = left pedal ganglion ablated.

pedal musculature was similar for snails with pedal commissurotomies and those with pedal plus cerebral commissurotomies (Fig. 3). Animals with both commissures transected initially showed less propodial expansion. Recovery began with the anterior portion of the foot and progressed posteriorly.

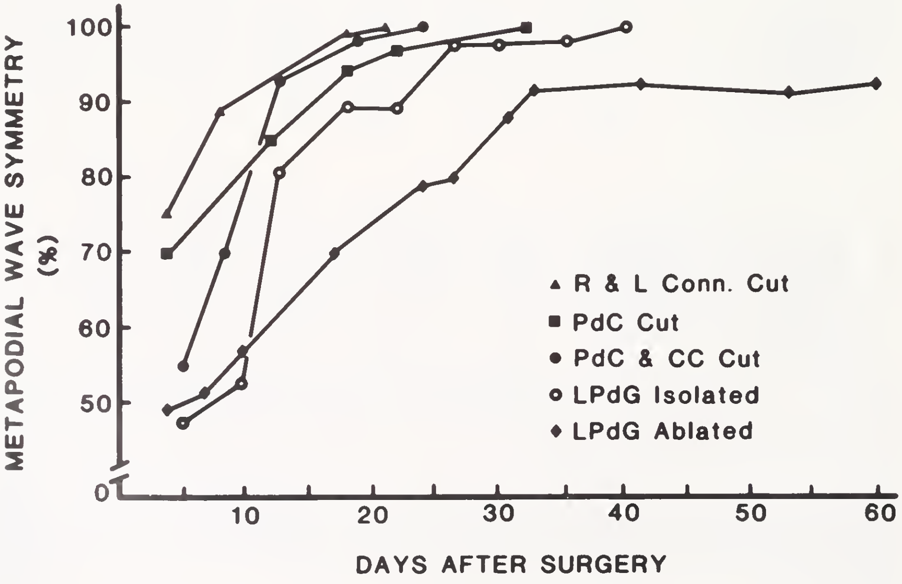

FIGURE 3. Recovery of metapodial wave symmetry in the five experimental groups. Symbols and abbreviations given in Figure 2.

### *Left pedal ganglion isolated*

The ganglion was held in place by its pedal nerves after its central connections were cut. The right side of the foot functioned normally, but the left half of the metapodium failed to participate in locomotion. One animal also exhibited abnormal coordination of a small portion of the right posterior metapodium. The six snails that received this lesion were slower to begin crawling than snails with previously described operations (Fig. 2). The left propodium and the anterior-most portion of the metapodium were the areas in which recovery was first seen. Control over pedal musculature was regained in an anterior to posterior progression, and complete pedal waves were seen on the operated left side in all 6 animals by day 40.

### *Left pedal ganglion ablated*

The most severe operation performed in terms of CNS damage was excision of an entire pedal ganglion (Fig. 1E). Behavior was similar to that following ganglion isolation. All 25 snails were crawling by day 5, although pedal musculature on the left side was inactive and the right posterior metapodial tip was also inactive in one-fourth of the snails. Recovery resembled that following left pedal ganglion isolation, except that it progressed more slowly and only 5 of 25 snails exhibited complete recovery (Fig. 3). The remaining snails all had some region on the left side of the foot that failed to be activated when tested on postoperative day 60.

## Discussion

Neurons that carry controlling information from cerebral ganglia to pedal ganglia have been classified as "command" neurons in *Aplysia* (Jahan-Parwar and Fredman, 1978), *Pleurobranchaea* (Gillette *et al.,* 1978), and *Lymnaea* (McCrohan, 1984). When both cerebropleural connectives were cut in *A. californía,* normal crawling continued and only the escape locomotion was abolished (Jahan-Parwar and Fredman, 1979). If both cerebropedal connectives were cut, only limited movements were generated; locomotion stopped entirely when all four connectives were severed. Transection of cerebropedal connectives only slows the locomotor rate in *Clione* (Satterlie and Spencer, 1985). In *Melampus,* signals travelling through either the cerebropleural or cerebropedal connectives are sufficient to initiate locomotion (Snyder, 1986). In the work reported here, snails with bilateral connective transections did not emerge from their shells and crawl as early as the other groups. The timescale for their onset of locomotion could have allowed for connective regeneration (Snyder, 1986).

In addition to interactions between cerebral and pedal levels, side-to-side coordination is important for locomotion. When the pedal commissure was cut in *Clione* or *Aplysia,* the right and left sides of the animal moved asynchronously (Jahan-Parwar and Fredman, 1980; Arshavsky *et al.,* 1985a; Satterlie and Spencer, 1985). In *Melampus,* cutting the cerebral commissure alone had no detectable effect on locomotion (Moffett and Snyder, 1985), whereas cutting the pedal commissure alone was almost as devastating as cutting both commissures.

Ablation of a pedal ganglion results in the same central lesions as pedal ganglion isolation, but also includes loss of a large population of neurons. The degree of recovery we observed following this lesion was therefore quite remarkable, especially considering that the recovery occurred before new neurons are likely to have been generated (Snyder, 1986). For all operations, the rate of behavioral recovery was rapid, considering distance between ganglia and the fact that we had cut neural trunks rather than crushing them, so growing axons had no mechanical linkage to follow in finding their targets. Only animals which had the left pedal ganglion removed failed to regain complete control over pedal musculature.

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# THE CONTINUING ENIGMA OF CIGUATERA*

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## ABSTRACT

Research on ciguatera fish poisoning has expanded significantly over the last decade. In large part, this increase in effort is due to the identification of several benthic dinoflagellates as the toxin producers, a discovery soon followed by a series of field and laboratory studies on their distribution, abundance, growth characteristics, and toxin production. Equally important have been advances in the analytical techniques and equipment needed to chemically characterize the toxins. Much of that work benefited significantly from the rapid progress in chemical research on the numerous other toxins produced by marine dinoflagellates.

Despite this surge in activity (summarized in the proceedings of four recent conferences or workshops: Ragelis, 1984; Salvat, 1985; Anderson *et al.*, 1985; this issue), the general state of knowledge on ciguatera remains relatively poor, both in terms of toxin chemistry and the physiological ecology of the causative dinoflagellates. Some important generalizations are gaining acceptance, but discrepancies and disagreements abound. One of the objectives of this review is to place the many recent papers on ciguatera in a current perspective that not only identifies common observations or conclusions, but also accentuates those areas that require more research effort to resolve disagreements or contradictions.

## INTRODUCTION

In many tropical regions, it has long been known that consumption of certain coastal marine fishes can cause human illness and occasional death. The name "ciguatera" was given to this phenomenon by the Spanish, based on the belief that a marine turban snail (called "cigua" in the Caribbean) was responsible for poisoning settlers in Cuba. Reports of similar fish poisoning in the Pacific date back to the early 17th century (Banner, 1976). Today the term "ciguatera" refers to intoxications resulting from the ingestion of tropical and subtropical finfish, distinct from histaminic poisonings or those associated with the pufferfish (Halstead, 1967).

Morbidity statistics are highly unreliable due to the tendency of many individuals not to report such illnesses, the wide geographic distribution of many islands where the problem is endemic, and the variability in symptomology. Mean annual incidence of reported cases in the Pacific island region (excluding Hawaii and Australia) is about 1–4 cases per thousand population (Lewis, 1984; Yasumoto *et al.*, 1984). In the Caribbean, 4.2 cases per thousand were reported from St. Thomas in the Virgin Islands (Olsen *et al.*, 1984). These values can be scaled up by factors of 2–5 using estimates of the fraction of poisonings that are never reported. The resulting estimates and those from other affected areas indicate that ciguatera has been responsible for far more cases of human illness over the past eight years than any other kind of seafood toxicity associated with consumption of fresh marine organisms (Ragelis, 1984). As many as 10,000–50,000 individuals may be poisoned by ciguatera annually.

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* For reprints of the entire ciguatera workshop proceedings contact: New England Biolabs Foundation, P.O. Box 413, Wenham, MA 01984. Those interested in reprints of individual papers should contact the author directly.

Although rarely fatal, the intoxications can be extremely debilitating and in some cases can recur sporadically for years after the initial poisoning. However, the most important impact of ciguatera may well lie in its effects on small-scale fisheries for local consumption and for export. This is especially serious in those poor or densely populated islands where fish traditionally have been a primary source of protein. Reviews by Lewis (1984) and Olsen *et al.* (1984) examined the impacts of ciguatera on marine resource development in the Pacific and Caribbean regions, respectively. In both areas, ciguatera is considered one of the most important constraints to fisheries resource development, second only perhaps to the inadequate size of those resources relative to the additional demands expected with future population and economic growth.

The symptoms of ciguatera poisoning have been described in great detail in numerous publications (Bagnis, 1968; Bagnis *et al.,* 1979; Withers, 1982; Ragelis, 1984; Yasumoto *et al.,* 1984; Steidinger and Baden, 1985). Usually the illness begins with gastrointestinal inflammation, leading to severe dehydration and weakness and eventually cardiovascular and neurological distress. The most distinctive features of ciguatera are severe puritus, hot/cold reversal (the "dry ice sensation"), and tingling and numbness of the extremities. A distinctive feature of this illness is that the neurological symptoms can persist for months or even years, occasionally recurring in seemingly healthy individuals long after their recovery from the initial poisoning. It is also noteworthy that ciguatera symptoms are highly variable between individuals and between regions. These latter differences are due in part to the fishes consumed. For example, Bagnis (1968) associated gastrointestinal disorders with the consumption of herbivorous fish such as the surgeonfish, and cardiovascular and neurological symptoms with carnivores such as grouper or snapper. As will be discussed later, this polymorphism in clinical features indicates that several toxins are involved in ciguatera poisoning—some confined to the primary herbivore consumers and others being transferred through the food chain to the largest predators.

There are no established treatments for ciguatera patients, although injections of steroids, non respiratory depressants, antihistamines, antidiarrhetics, and vitamins seem to alleviate some of the symptoms (Yasumoto *et al.,* 1984). Native remedies involve treatments which rapidly purge the digestive tract (Lobel, 1979).

## THE DINOFLAGELLATE TOXIN SOURCE

Despite the long history of ciguatera, the most probable source of the toxins, namely a group of benthic dinoflagellates, was only discovered within the last decade. Even now there is a degree of uncertainty as to whether the toxins isolated from ciguatoxic fish are the same as those produced by cultures of these dinoflagellates.

Prior to these recent developments, many theories implicated diseased fish, pollution, and other general phenomena in the poisonings. An exceedingly thorough examination of the feeding behavior of ciguatoxic fish in the Pacific by Randall (1958) led to a food chain theory whereby the toxin was presumed to be produced by a benthic microorganism (an unspecified alga, protozoan, fungus, or bacterium) which is first ingested by herbivorous fishes; the toxin is then transferred to larger carnivores. This theory proved to be remarkably accurate, although nearly 20 years passed before its validity was proven by the identification of the source organisms. The breakthrough occurred when Yasumoto *et al.* (1977b) found considerable toxicity in a sample of algae and detritus collected from the surface of dead coral in the Gambier Islands of French Polynesia. They also found high numbers of a large dinoflagellate in the most toxic samples and relatively few in low toxicity samples. The same pattern held for the stomach contents of high and low toxicity fish. Tentatively identified as

*Diplopsalis* sp., the organism was later placed in a new genus and named *Gambierdiscus toxicus* (Adachi and Fukuyo, 1979). To confirm the link between this dinoflagellate and fish toxicity, Yasumoto *et al.* (1977b) used various sieving and separation techniques to obtain dinoflagellate-rich fractions from heterogeneous detrital samples containing sand and coral fragments. Bioassays of the dinoflagellate samples showed that toxin content was directly proportional to the number of *G. toxicus* in the samples. Extracts from the dinoflagellate samples yielded two toxins, one with chemical and pharmacological properties identical, or closely related to ciguatoxin and the other resembling maitotoxin.

This study seemed to fix conclusively *G. toxicus* as the ciguatera elaborator, but the presence of many other co-occurring benthic dinoflagellates in the toxic samples and the detection of minor toxins of unknown origin in grazing herbivores and detritus feeders led Yasumoto *et al.* (1980) to test other dinoflagellates for toxicity. The results of their work and that of others (Nakajima *et al.*, 1981; Tindall *et al.*, 1984; Yasumoto, 1987) document the surprising fact that many of the dinoflagellates in tropical waters that live on or in close association with macroalgae or other surfaces are toxic. An example of the unexpected nature of these findings is that a survey of benthic dinoflagellates from Okinawa revealed toxins in all nine of the species examined (Nakajima *et al.*, 1981). Such results would never be expected in a similar survey of planktonic dinoflagellates, where toxicity is by far the exception rather than the norm. These results also add a degree of confusion to the ciguatera problem, since the existence of an array of toxins within an assemblage of organisms necessarily confounds the interpretation of chemical analyses and epidemiological surveys.

Three different types of toxins have been detected in the benthic dinoflagellates. *Gambierdiscus toxicus, Prorocentrum lima, P. concavum, Ostreopsis siamensis, O. ovata, Amphidinium carteri,* and *A. klebsii* all produce toxins which can kill mice (Nakajima *et al.*, 1981; Yasumoto, 1987). *Amphidinium carterii, A. klebsii, Coolia monotis,* and *P. rhathymum* (= *mexicanum*) produce toxins with strong hemolytic activity, but in fact some degree of hemolysis was observed using extracts of all nine species examined by Nakajima *et al.* (1981). *Prorocentrum concavum, A. carterii,* and *A. klebsii* produce strong ichthyotoxins; *P. concavum* is exceptionally potent. It should be stressed that not all of these toxins are involved in ciguatera. Although ichthyotoxins and hemolytic agents could have important effects on fish in tropical areas, only the species that produce toxins capable of killing mice will be considered further.

All of the work described above was based on cultures of dinoflagellates from the southern Pacific region. Subsequent investigations by Shimizu *et al.* (1982) confirmed the presence of *G. toxicus* in Hawaii. Similarly, surveys in the Caribbean by Tindall *et al.* (1984) indicated a species assemblage the same as that in the Pacific, including *G. toxicus* which produces ciguatoxin, one other lipid-soluble toxin, and maitotoxin. *Prorocentrum concavum* extracts were actually more potent than those from *G. toxicus* in that study.

The overall view that arises is that the benthic dinoflagellate community described above can be found throughout the world in tropical and subtropical regions where ciguatera is a problem. It is a diverse community consisting of species from at least four genera. All are photosynthetic, but they have little else in common other than their association with the benthos. Even within the benthos, they differ greatly in their habitat preference with some living attached to macroalgae and other surfaces, some in the sand, and the remainder free-swimming but still closely associated with surfaces. The reason that so many of these benthic dinoflagellates are toxic is a fascinating mystery that may be linked somehow to their habitat preference. Although this is a clue that bears on the origins and functions of these toxins, elucidation of their role in dinoflagellate metabolism remains a distant but tantalizing goal.

## TOXIN CHEMISTRY

Despite a concerted research effort over more than two decades, knowledge of the chemical characteristics and structure of the ciguatera toxins is incomplete. Reasons for this status are many: the toxins are present in extremely low concentrations in fish tissue; they can be unstable during the complex extraction and purification procedures; production of ciguatoxin in dinoflagellate cultures has been either minimal or non-existent; and reliable, sensitive assay methods specific for each toxin are not available.

The principal toxin in ciguatera poisoning is called ciguatoxin. This was first purified from red snapper in the Pacific (Scheuer *et al.,* 1967) and later from moray eel and shark flesh. Moray eel liver has been used extensively in subsequent studies because of its relatively high toxin content. The yield after extraction is still extremely low, however, as initial concentrations average only 10–20 ppb (Yasumoto *et al.,* 1984; Tachibana *et al.,* 1987). Ciguatoxin is insoluble in water or benzene, but readily partitions with methanol, acetone, ethanol, or 2-propanol.

The molecular structure of ciguatoxin has not yet been established, although $^1H$ NMR data suggest a molecular weight of 1111.7 ± 0.3 amu and a formula similar to $C_{53}H_{77}NO_{24}$ or $C_{54}H_{78}O_{24}$ (Tachibana *et al.,* 1987). The most probable configuration is that of a highly oxygenated long-chain fatty acid in which most of the oxygen atoms occur as cyclic ether linkages. This latter observation is consistent with the similar behavior of ciguatoxin and okadaic acid in thin layer chromatography (Murakami *et al.,* 1982) and with the cross-reaction of ciguatoxin and other polyether toxins in immunoassays (Baden *et al.,* in prep.; Hokama *et al.,* 1987).

Two additional toxins can be extracted from ciguatoxic fish, one of which is ether-soluble like ciguatoxin and the other water-soluble. The former has been called scaritoxin (Bagnis *et al.,* 1974) because it is found predominantly in many species of parrotfish (*Scarus*). It is easily separated from ciguatoxin on a DEAE-cellulose column and migrates differently in thin layer chromatography (Chungue *et al.,* 1977). No scaritoxin could be detected in the diet of the parrotfish (Yasumoto *et al.,* 1977a), yet flesh samples clearly contained the toxin. The presence of ciguatoxin as the dominant toxin in the gut and liver of the parrotfish was a further indication that scaritoxin is not a naturally occurring toxin in the fish's diet but instead is a metabolite of ciguatoxin (Yasumoto *et al.,* 1977a). This hypothesis was recently confirmed by the demonstration that ciguatoxin and scaritoxin can be reversibly interconverted by manipulation on basic alumina columns (Tachibana *et al.,* 1987).

Looking back to the assumed polyether structure of ciguatoxin with its many hydroxyl groups, it now seems reasonable that hydrogen-bonding at various locations could yield compounds with distinct chromatographic and pharmacological characteristics but the same general structure as the parent ciguatoxin (Tachibana *et al.,* 1987). Such changes could readily occur within fish following consumption of the dinoflagellate.

The second major toxin involved in ciguatera poisonings is maitotoxin, originally isolated from the surgeonfish *Ctenochaetus striatus* (Tahitian name, "maito") and subsequently found in significant quantities in extracts of cultures of *G. toxicus* and possibly *P. concavum* (Tindall *et al.,* 1984). Maitotoxin is more polar than ciguatoxin and is thus soluble in water. Its occurrence is thus limited to the viscera of herbivores or benthic grazers in contrast to the lipid-soluble ciguatoxin which can accumulate in flesh and move through the food chain. Although maitotoxin is produced in abundance in dinoflagellate cultures, it remains poorly characterized. Purified material yields an amorphorus white solid whose molecular weight is thought to be around 3300 amu (Yasumoto, 1987). There are no amino acid or fatty acid moities in the molecule, and there appear to be no chemical similarities between maitotoxin and ciguatoxin (Yasumoto *et al.*, 1984).

A third toxin which may be involved in ciguatera poisonings is okadaic acid, a polyether fatty acid derivative first found in sponges. This lipid-soluble compound has been isolated from *P. lima* (Murakami *et al.*, 1982), a dinoflagellate included in the benthic ciguatera community. Okadaic acid and structurally similar compounds have been implicated in diarrhetic shellfish poisoning (DSP), most commonly due to planktonic dinoflagellates of the genus *Dinophysis* (Yasumoto, 1985). Symptoms following the consumption of shellfish containing these compounds include diarrhea, vomiting, and other gastrointestinal disorders. Since similar symptoms have been reported for some ciguatera poisonings and since *P. lima* is present in the seaweeds grazed by herbivorous tropical fish, it is possible that okadaic acid is causing one type of illness among several grouped under the general term "ciguatera."

One intriguing aspect of recent work on toxin chemistry is that ciguatoxin production has been extremely low in laboratory cultures of *G. toxicus*, even when strains isolated from highly toxic wild material are used (Yasumoto *et al.*, 1979b; Bagnis *et al.*, 1980). A lack of detectable ciguatoxin in wild *G. toxicus* populations also has been observed (Gillespie *et al.*, 1985). There are numerous reports of lipid-soluble material from culture extracts that kill mice (*e.g.*, Yasumoto *et al.*, 1977, 1979b; Withers, 1982; Tindall *et al.*, 1984; Durand-Clement, 1987), but the lack of assay methods that distinguish ciguatoxin from maitotoxin leaves a cloud of uncertainty over such results. Some workers believe that traces of maitotoxin can remain in the lipid soluble "ciguatoxin" fraction and thus result in mouse mortality even when ciguatoxin is absent (Gillespie, pers. comm.). Ciguatoxin is readily separated from maitotoxin through the use of a silicic acid column and a stepwise elution with chloroform and methanol (Tachibana, 1980). Ciguatoxin elutes with chloroform: methanol at 9:1 and maitotoxin at 1:1. This procedure has not been used routinely by all workers, however, so the problem of residual maitotoxin remains a potentially important artifact in many studies. A more complicated and cautious approach to studies of this kind is that of Baden *et al.* (1985), who supported their claim of production of a ciguatoxin-like compound in *G. toxicus* cultures by demonstrating that their lipid-soluble extract contained a sodium channel depolarizing toxin whose effect on a crayfish giant axon could be partially blocked by tetrototoxin. This type of assay, or the column separation scheme described above, would seem to be necessary prerequisites for all work directed at the characterization or measurement of ciguatoxin; yet such has not generally been the case.

The state of the chemical characterization of the ciguatera toxins can be summarized as follows. It is clear that several toxins may be responsible for the poisonings. Ciguatoxin, the primary toxin, has been isolated from larger carnivores, but is only partially characterized because of an inadequate supply of purified material. Although considerable circumstantial evidence has been compiled linking *G. toxicus* to this toxin, it has not yet been conclusively demonstrated that the toxin produced by the dinoflagellate is either identical to, or is a direct precursor to the ciguatoxin accumulating in the fish. Scaritoxin, another lipid-soluble toxin detectable in fish flesh is presumably a metabolite of ciguatoxin, apparently formed after the fish has ingested the primary toxin. Maitotoxin is the most readily available toxin since it is produced in abundance in *G. toxicus* cultures, yet its chemical structure also remains unknown. The extremely high potency of maitotoxin, and the likelihood that trace quantities of it remain in the lipid fraction of many separation schemes, makes it difficult to interpret earlier studies, especially those claiming ciguatoxin production in *G. toxicus* cultures. References to extracted compounds as "ciguatoxin-like" or "maitotoxin-like" abound in the current literature, underscoring the analytical uncertainties that remain in this field despite years of concerted research effort. Okadaic acid, the final toxin of concern here, has been well-characterized chemically but has not been shown to be directly involved in fish poisonings. Its inclusion in this discussion is based on the similarities between symptoms associated with this toxin as a cause of diarrhetic shellfish poisonings and those from certain ciguatera poisonings, as well as on the proven production of this compound by *P. lima*, a prominent species within the ciguatera dinoflagellate community.

## PHARMACOLOGY

The suite of symptoms associated with ciguatera poisonings is due in part to the wide variety of fishes consumed and the diversity of toxins within those fishes. In addition, pharmacological studies on extracted toxins are subject to the same artifacts discussed earlier due to variability in sample purity. Nevertheless, a coherent picture of the effects of the ciguatera toxins on living systems is beginning to emerge.

Both ciguatoxin and maitotoxin are among the most potent marine toxins known, having $LD_{50}$'s of 0.45 and 0.13 $\mu g\ kg^{-1}$ [intraperitoneal (i.p.), mouse] respectively (Tachibana, 1980; Yasumoto, 1985). Bagnis *et al.* (1987) used bioassays of leftover portions of fish that had caused ciguatera poisonings to derive a relationship between oral dose and ciguatera symptoms in humans. The extreme potency of ciguatoxin determined from intraperitoneal injections in mice was still evident in terms of human oral potency, with a mean dose for 50% illness at 2 $ng\ kg^{-1}$ and a minimum lethal dose estimated to be 20 $ng\ kg^{-1}$. The primary action of ciguatoxin now appears to be a depolarization of the sodium channel, an effect that can be blocked by application of tetrototoxin (Rayner 1970; Rayner and Kosaki, 1970; LeGrand and Bagnis, 1984). Scaritoxin also has been shown to have a depolarizing action on excitable membranes and generally seems to have a pharmacological mode of action close to that of ciguatoxin. In hindsight, this is to be expected since it is now clear that the two compounds are structurally related. Li (1965) reported that ciguatoxin isolated from several fish species functioned as an anticholinesterase, but this contention was tested by Rayner *et al.* (1969) who concluded that there may be some inhibition of cholinesterase in *in vitro* preparations but that this was not an effect of ciguatoxin in living organisms.

Maitotoxin also acts as a neurotoxin, but its effects are most probably centered on the calcium channel. Neurophysiological studies (Takahashi *et al.*, 1982, 1983; Ohizumi *et al.*, 1985; Miller and Tindall, 1985; Ohizumi, 1987) indicate that maitotoxin causes positive inotropic effect on smooth muscle, suggesting that the toxin causes an increase in $Ca^{2+}$ permeability, probably through calcium channels. This action is not affected by treatment with tetrototoxin or by excess sodium.

The same functions that make the ciguatera toxins potent marine poisons also makes them potential tools in the study of excitable membranes. The utility of saxitoxin and tetrototoxin as molecular probes is already well established (Caterall, 1985), but the active use of ciguatoxin and maitotoxin in similar neurophysiological studies only awaits the increased availability of purified material.

## ASSAY METHODS

Ciguatera toxins are odorless, tasteless, and generally undetectable by any simple chemical test, so bioassays traditionally have been used to monitor suspect fish. Many native tests for toxicity in fish have been examined, including discolorations of silver coins or copper wire or the repulsion of flies and ants, but all of these were rejected as invalid (Banner, 1964).

Oral feeding of fish to cats is a simple and sensitive assay, but has the disadvantage that the cats often regurgitate part of the meal. Since the mongoose does not regurgitate and thus exhibits a response that is related to the amount of toxin ingested, a roughly quantitative assay was designed and used extensively in Hawaii using trapped wild animals (Banner, 1976).

Feeding tests such as those above are useful in screening fish for toxicity, but they are non-quantitative and cumbersome. As is common with other dinoflagellate toxins, a mouse bioassay was developed, but this procedure required purification of fish extracts since mice are relatively insensitive to ciguatoxin (Yasumoto *et al.*, 1971). The mouse bioassay has been used in numerous surveys in the Pacific and is described in detail in Yasumoto *et al.* (1984).

One alternative to the use of mice is the mosquito bioassay which was recently used by Bagnis *et al.* (1987) to obtain a dose-response relationship between ingested ciguatoxin and clinical symptoms in man. The mosquito assay correlates reasonably well with cat and mouse bioassays, and has the additional advantages that it is rapid, dependent on a simple extraction, and requires only a small amount of fish for analysis.

All bioassay methods have common disadvantages, perhaps the most important of which is the lack of specificity for individual toxins. Several alternative methods are now under development that have the potential to provide the needed sensitivity and specificity. One is a radioimmunoassay for ciguatoxin originally developed in Hawaii (Hokama *et al.*, 1977). During a two-year study, this method was used to screen amberjacks (*Seriola dumerili*) on the Hawaiian market, 15% of which were rejected (Kimura *et al.*, 1982). No poisonings were reported from that fish species during the study, although other untested species did cause illness. Despite this success, the radioimmunoassay is too costly and time-consuming for routine use and does cross-react with okadaic acid and other polyether compounds. An inexpensive, rapid colorimetric enzyme immunoassay was then developed (Hokama *et al.*, 1983) which was subsequently adapted further to what is now called the "stick test" (Hokama *et al.*, 1987). This technique, which uses small, coated bamboo sticks to assay the fish flesh, shows great promise since each assay takes less than 15 minutes and the procedures are sufficiently simple to be employed in the field. One disadvantage, however, is that the antibody reacts with okadaic acid, brevetoxin, and other polyether compounds with structures similar to ciguatoxin. It is hoped that ongoing attempts to develop monoclonal antibodies to each of these closely related polyethers will allow the "stick test" to attain the necessary degree of specificity. The importance of this assay should not be discounted even in its present form, however, since the cross-reaction problems seem to generate false positives (*i.e.*, rejection of fish that are safe to eat) but very few false negatives. This clearly seems to be the direction of choice for future work on assay development.

## FIELD ECOLOGY

### *Dinoflagellate/host specificity*

The ciguatera dinoflagellates are all considered benthic, epiphytic, or metaphytic—living attached to or in close association with sand, coral, macroalgae, and other surfaces. Table I lists the macroalgal species found associated with high concentrations of *G. toxicus*. Most of these host algae are branched or tufted in form as sug-

TABLE I

*Macroalga genera with epiphytic* Gambierdiscus toxicus[1]

| | |
|---|---|
| Green algae | Red algae |
| CHLOROPHYTA | RHODOPHYTA |
| *Caulerpa* | *Acanthophora* |
| *Chaetomorpha* | *Amphiroa* |
| *Cladophora* | *Asparagopsis* |
| *Codium* | *Digenia* |
| | *Galaxura* |
| Brown algae | *Gelidium* |
| PHAEOPHYTA | *Hypnea* |
| *Dictyota* | *Jania* |
| *Sargassum* | *Laurencia* |
| *Turbinaria* | *Pterocladia* |
| | *Spyridea* |

[1] Data compiled from the Caribbean and tropical Pacific from: Yasumoto *et al.*, 1977; Shimizu *et al.*, 1982; Whithers 1982; Taylor and Gustavson, in press; Carlson *et al.*, 1984; Carlson and Tindall, 1985; Carlson 1984; Taylor 1985; Bagnis *et al.*, 1985; Gillespie *et al.*, 1985.

gested by Taylor (1985), but *G. toxicus* will also attach to most kinds of algae regardless of structure (Gillespie *et al.*, 1985) while avoiding bare coral substrate and seagrass blades (Carlson and Tindall, 1984).

Algae which persist on coral reefs in the presence of herbivores usually are structurally tough or distasteful (*e.g., Halimeda, Penicillus, Caulerpa,* etc.). Delicate filamentous algae which are readily eaten by many herbivorous fishes are rare and usually appear on new bare patches of rock and coral (*e.g., Polysiphonia, Enteromorpha,* etc.). Because filamentous algae are rare, they have been thus far undersampled for *G. toxicus* occurrence. Randall's (1958) early insight into ciguatera ecology considered whether outbreaks occur when reef surfaces were bare. The question remains whether under these circumstances the first colonizing filamentous algae might also be epiphytized by *G. toxicus.* Many of the host algae in Table I persist as macrophytes either by living in habitats or zones where herbivory is low or by producing secondary metabolites which inhibit fish feeding (Hay 1984, 1985; Hay and Goertemiller, 1983).

Therefore, the occurrence of *G. toxicus* on certain of these macroalgae may not be a good indicator of their importance in the transfer of toxins to higher trophic levels. The transfer actually may occur through grazing on the less abundant, undersampled macroalgae which are preferred foods. In other words, the persistence of macroalgae with epiphytic *G. toxicus* may only be an indication of what is *not* being eaten, with the real uptake of the toxin occurring as less abundant, smaller algae are cropped by the herbivores. This is analagous to the nutrition of phytoplankton in the central oceans where essential nutrients like nitrogen and phosphorus are below analytical detection limits but are, nevertheless, available through rapid recycling or small-scale patchiness. Clearly, despite the numerous studies which have enumerated the host macroalgae for the epiphytic dinoflagellates, an understanding of the reasons for these associations is far from complete. Suggestions of host selectivity based on form and structure may be valid (Taylor, 1985; Taylor and Gustavson, in press) but must remain speculation until controlled experiments are conducted.

### *Fish herbivory*

Herbivorous fishes comprise a diverse taxonomic assemblage of species and possess widely different capabilities for utilizing plants as food (Lobel, 1981). An important uncertainty is the relationship between ciguatera toxicity and fish digestive mechanisms and feeding selectivity.

Certain herbivorous fishes are well-known for morphological specializations enabling trituration, such as parrotfishes (Scaridae) with a bony pharyngeal mill and certain surgeonfishes (Acanthuridae, *e.g., Ctenochaetus* spp.) with a muscularized, gizzard-like stomach. Another mechanism used by some marine fishes for rupturing ingested plant cells—lysis by gastric acidity (pH range 2.4–4.3)—recently has been described (Lobel 1981). Fishes with acidic stomachs include certain surgeonfishes of the genus *Acanthurus* and the territorial herbivorous damselfishes [Pomacentridae; *Stegastes* (= *Eupomacentrus*) spp.]. Utilization of plant foods by fishes is apparently limited to these three digestive mechanisms. Fishes are not known to produce cellulase or other enzymes capable of digesting plant cell walls. However, they do produce several carbohydrases capable of digesting plant cell contents (Kapoor *et al.,* 1975). An intestinal microorganism has been found recently in the gut of two herbivorous fishes in the Red Sea but was absent from the guts of several other species of the same family (Acanthuridae; Fishelson *et al.,* 1985). This microorganism probably does not have a primary role in digestion (Fishelson *et al.,* 1985) and none have been identified in other herbivorous fishes (Kapoor *et al.,* 1975).

Herbivorous fishes are classed as "browsers" or "grazers" (Jones, 1968). Grazers ingest substantial quantities of sand and coral particles while feeding on algae by either rasping the substrate or sucking loose grains. Browsers bite or tear algae and rarely ingest any inorganic material. Herbivorous marine fishes are further characterized by three general types of alimentary morphology: (1) an elastic stomach capable of secreting strong acids (pH 2.4–4.3), with a long intestine, (2) a thick-walled, gizzard-like stomach (pH 6.3–7.9) and a medium length intestine and, (3) a bony pharyngeal mill with no stomach present (anterior intestine pH $\sim$ 8.4) and a relatively short intestine (Lobel, 1981). The gizzard-like stomach and the pharyngeal mill are characteristic of grazers. Fishes with an acidic stomach are browsers. For details see Lobel (1980, 1981) and Lobel and Ogden (1981).

It is unknown how these different digestive capabilities may relate to ciguatera toxicity, but it has been shown that the surgeonfish, *Ctenochaetus striatus* (type 2) and parrotfish species (type 3) have distinct toxin characteristics as described previously (Bagnis *et al.,* 1974; Yasumoto *et al.,* 1984). These fishes are also the most frequently implicated in ciguatera poisoning while fishes belonging to type 1, such as the Pacific surgeonfish, *Acanthurus triostegus,* are of lower risk. The relationships between this pattern and the fishes' feeding habits or the possible interaction of ingested dinoflagellate and fish gut chemistries remain obscure.

Few studies have quantified the preference by fishes for particular algal species (reviewed by Ogden and Lobel, 1978). It has been more common to assess survivorship of transplanted algae exposed to the ensemble of reef herbivores (*e.g.,* Earle, 1972; Hay, 1984; 1985). Analysis of stomach contents in herbivorous fishes is difficult because some species completely grind their food. Gut contents show only what has been eaten, do not necessarily reflect preferences, and can be further confounded by the relative indigestability of some algae over others. Nevertheless, many studies show that certain algae are much more likely to be eaten than others, including several known hosts to *G. toxicus* (Table I; Earle, 1972; Ogden and Lobel, 1978; Hay, 1984; 1985).

Browsers consume fine filamentous algae and epiphytes. Fishes of this type include the surgeonfish *Acanthurus triostegus* (Acanthuridae, Randall 1961) and the territorial damselfishes, *Stegastes* spp. (Pomacentridae, Lobel 1980). These damselfishes feed specifically on epiphytes overgrowing small red algal thalli (Lobel, 1980).

TABLE II

*Predator consumption of herbivorous fishes in the Caribbean*[1]

| Herbivore | % predator spp. having eaten this prey (n = 58 spp) | % of fish individuals eaten by all predators (n = 391 ind.) |
|---|---|---|
| Grazers | | |
| Scaridae | 28% | 13.3% |
| Monocanthidae | 21% | 3.8% |
| Acanthuridae | 14% | 2.3% |
| Browsers | | |
| Pomacentridae | 10% | 3.4% |

[1] Data from Randall, 1967.

These fishes have not yet been assayed for ciguatera toxicity probably because damselfishes are small and not eaten by people. It seems, however, that they are prime candidates because *G. toxicus* is frequently epiphytic on red algae, and they are important in the trophic linkage to higher carnivores (Table II).

Grazers with a gizzard-like stomach consume microalgae mixed with fine sand and detritus. This group includes the herbivorous fish, *Ctenochactus* spp., frequently implicated in ciguatera fish poisoning (Randall, 1980). Some species of this group have solid cropping teeth and are able to bite a variety of small algal thalli, but *Ctenochaetus* is distinct. This fish has numerous, very elongate teeth with expanded incurved tips which are loosely attached in the jaw (Randall, 1955). *Ctenochaetus* spp. feed on fine particulate material. *C. strigosus* in Hawaii contained up to 90% fine inorganic sediment with the rest being unicellular algae, small fragments of filamentous algae, and detritus (Randall, 1955; Jones, 1968). Randall (1955) related the following account of *C. strigosus* feeding: "When a thallus of fine filamentous red algae (*Polysiphonia* sp.) was placed in an aquarium . . . the fish attempted to feed upon it. Their slender movable teeth, not able to effectively bite off pieces, soon became entangled in the alga, resulting in very little being ingested." However, the fish was able to feed on fine particles of the alga that settled on the bottom. It sucked up particulate algae with very fine sediment. Large sand grains were generally avoided. According to Carlson and Tindall (1985), *G. toxicus* is rarely found on sand, thus it would seem that if it is eaten by *Ctenochaetus,* it must be sucked off the surfaces of macroalgae.

The herbivorous fishes having a bony pharyngeal mill are parrotfishes (Scaridae). Adult reef parrotfish graze algae overgrowing dead coral surfaces and ingest quantities of calcium carbonate with their algal food (Randall, 1967, 1974; Ogden, 1977). Juvenile parrotfish scrape fine filamentous algae and epiphytes from a variety of surfaces. When possible, they will feed on most kinds of the algae (*e.g.,* Earle, 1972) on which *G. toxicus* is epiphytic (Table II). Thus, despite the toughness or "bad taste" of certain host macroalgae, *G. toxicus* could be removed from these surfaces by parrotfishes. Controlled feeding preference experiments have demonstrated that one species, *Sparisoma radians,* will eat seagrass blades with epiphytes in preference to bare blades (Lobel and Ogden, 1981). Parrotfish are the dominant family by weight on many tropical reefs and are the most common herbivore prey of large piscivores (Table I. Randall, 1967, 1974).

Once again generalizations concerning ciguatoxic fishes and their food habits are not yet possible given available field data. It is clear that numerous herbivores can be toxic and that the toxin can easily move to higher food chain levels through predation. What is unclear is how the toxin is obtained by herbivores with such varied feeding habits and preferences. Perhaps the colonization of many different algal surfaces by *G. toxicus* ensures that it will enter the food chain through herbivorous fishes. But whether this happens as a continuous process, or sporadically when *G. toxicus* occurs on certain algae which rapidly colonize new reef surfaces and are then eaten by fishes, remains moot.

### *General habitat*

As discussed earlier, the ciguatera dinoflagellates are found in tropical waters throughout the world, but there is a general pattern to this distribution. In both the Pacific and the Caribbean, for example, ciguatera seems to be restricted to islands and is not found along continental margins. It is also apparently lacking in the waters of the islands of the Western Pacific (Banner, 1976). Exceptions to this generality include Florida and the Great Barrier Reef of Australia. However, the region of Florida that is affected is along the Keys and eastern coast which are subject to intrusions of oceanic water; in Australia, toxic fish are found predominantly around the offshore reefs and not along the continental margin (Banner, 1976). This general "oceanic" scenario was confirmed in a survey of 86 locations on 15 Caribbean islands by Taylor and Gustavson (in press), who generalized that *G. toxicus* is absent from nearshore localities on large, high islands or major land masses with substantial land runoff, but thrives in areas most exposed to oceanic waters, notably near offshore outcrops or on the windward side of islands.

Within a region where the ciguatera community occurs, certain generalizations are emerging as to habitat preference, but interestingly, these generalizations sometimes differ between the Pacific and the Caribbean. For example, based on numerous surveys of islands in the Pacific, Yasumoto and co-workers (1979a, 1980) indicate that *G. toxicus* was most abundant in relatively high energy environments—exposed reef areas and turbulent channels. In contrast, an extensive survey in the Caribbean by Carlson (1985) showed much greater abundance of this species in protected lagoons and other inshore stations compared to reef stations. This observation seems to conflict with other reports that reef fishes in that region are very toxic, but the close proximity of reefs and lagoons in the Virgin Islands allows fishes to move freely between the two locations for feeding.

There are several possible reasons for the disparity in habitat preferences described above. Inadequate sampling might be one explanation, since the epiphytic dinoflagellates are notoriously patchy even on spatial scales of a few meters (Yasumoto *et al.*, 1979a; Taylor and Gustavson, in press). Another factor might be related to the season of the sampling, since it is now a relatively common observation (discussed below) that dinoflagellate abundance can vary significantly over the year at certain stations, especially those exposed to storm and wave activity. Surveys conducted over a short interval at one time of the year might not be representative of the species distribution at other times. Whatever the reason for this discrepancy in habitat preference, it is clear that accurate descriptions of the field distribution of the ciguatera dinoflagellates are difficult to obtain, but are nevertheless extremely important.

There is general agreement on other aspects of the field distributions of *G. toxicus*. Workers in both the Pacific and the Caribbean have observed that *G. toxicus* does not occur at shallow depths or in areas with high light intensities (Yasumoto, 1978; Yasumoto *et al.*, 1980; Carlson, 1985; Taylor and Gustavson, in press). Carlson (1984) found that macroalgal-associated dinoflagellates were generally not found at depths less than 0.5 m where light levels exceeded $6.5 \times 10^4$ lux. Furthermore, dinoflagellate abundance was low in areas with white, sandy bottoms where light reflected from the bottom nearly equaled the incident irradiance. There is also general agreement that *G. toxicus* prefers high salinity water, being very scarce near the mouths of rivers or in areas of high runoff (Yasumoto *et al.*, 1980; Carlson, 1985; Taylor, 1985).

Long and short-term fluctuations have been observed in both the incidence of fish poisonings (Halstead, 1967; Banner, 1976) and the abundance of the ciguatera dinoflagellates. In Australia, *G. toxicus* cell numbers were shown to increase dramatically in September and October during two years of observations (Gillespie *et al.*, 1985). In the Virgin Islands, a similar periodicity in cell number was observed, with two peaks in abundance during twelve months of data (Carlson and Tindall, 1985). Although their data were more qualitative, Taylor and Gustavson (in press) noted seasonal fluctuations in *G. toxicus* abundance in Barbados. Relatively few environmental parameters were monitored during these studies, so it is difficult to speculate on the cause of the cyclical abundance. In all cases, however, low dinoflagellate abundance occurred during the periods when storm and wave activity were maximal. Stresses from wind and waves on the macroalgae are clearly reflected in the abundance of the dinoflagellate epiphytes. Carlson and Tindall (1985) also found a strong positive correlation between fluctuations in the numbers of toxic benthic dinoflagellates (including *G. toxicus*) and Virgin Islands' rainfall.

Several workers have looked for correlations between the fluctuating abundance of the ciguatera dinoflagellates and major nutrient concentrations, but without success. Yasumoto *et al.* (1980) found no relationship between inorganic phosphorus, total phosphorus, nitrite, nitrate, silicate, iron, dissolved organic carbon, and vitamin $B_{12}$ and *G. toxicus* cell concentrations in French Polynesia. These water samples were taken in the general vicinity of the macroalgae used for the *G. toxicus* counts. Carlson (1984) did nutrient analyses on water collected immediately adjacent to the macroalgae. Both phosphates and nitrates were significantly correlated with three predominant dinoflagellates (*G. toxicus, P. concavum,* and *P. lima*), but no single limiting nutrient was identified. These results are consistent with the view that these epiphytic dinoflagellates may specifically associate with macroalgae where high concentrations of nutrients are available for growth (Steidinger, 1983).

One popular notion about ciguatera is that it can arise in previously unaffected areas or become worse in areas with a long history of low-level toxicity—all in response to disruption or destruction of reef surfaces (reviewed by Randall, 1958; Banner, 1976). The concept is that freshly denuded surfaces on a reef are colonized by certain opportunistic species of macroalgae that are ideal hosts for the epiphytic dinoflagellates. Thus dredging, shipwrecks, hurricanes, and other man-made or natural disturbances can all create the new surfaces needed for colonization. Support for this theory comes from Cooper (1964) who related toxicity in the Gilbert Islands to the locations of wrecks and anchorages, by Bagnis (1969) who reported an outbreak of poisonings at the previously non-toxic atoll of Hao after major changes to the reef system, and by Bagnis *et al.* (1985) who document a decrease in toxicity in the Gambier Islands in the years following an initial flare-up which followed soon after major reef destruction. There are many other reports that support this hypothesized link between "new surfaces" and toxicity, but there are also many instances where such events were not associated with increases in toxicity. Banner (1974) points out that the blasting of channels in the Gilbert Islands, typhoon flooding in Fiji, dredging at Johnston Atoll, and even reef devastation by the starfish *Acanthaster* were not followed by toxicity. Free or "new" coral surfaces may well provide an excellent mechanism for the accumulation of ciguatera dinoflagellates, but there are clearly other factors that must be suitable as well if an outbreak is to occur.

## Culture Studies

Several ciguatera dinoflagellates have been studied in laboratory cultures, but the results obtained by different workers are sometimes confusing and contradictory. There is general agreement, however, on the temperature tolerance of *G. toxicus,* the only species for which data are available. Pacific strains of *G. toxicus* grow optimally near 27°C (Yasumoto *et al.,* 1984; Durand-Clement, 1987) but stop growing or die at temperatures above or below 30 and 20°C, respectively. The light tolerance of *G. toxicus* was examined by Yasumoto (1978) and Durand-Clement (1987) and interestingly, the relatively low light intensities reported for optimal growth (about 4000 lux) are very similar to field measurements of the light environment in Caribbean lagoons where Carlson (1984) found the highest numbers of this species.

Durand-Clement (1987) reports that *G. toxicus* growth was very poor in continuous light, but Carlson *et al.* (1984) successfully used continuous light for all of their culture studies on the ciguatera community. Similar discrepancies arise when growth data from bacteria-free cultures are compared. Durand-Clement (1987) found enhanced growth of *G. toxicus* (and a substantial decrease in the normally copious mucilage production) when bacteria were eliminated. However, Yasumoto *et al.* (1984) and Hurtel *et al.* (1979) both report that growth of this same species was markedly retarded in axenic culture, a finding similar to that of Carlson (1984) for *P. concavum.*

The benefits accruing to the ciguatera dinoflagellates from their close association with macroalgae remain entirely speculative and include enhanced nutrient availability, shading from dangerously high light intensities, and protection from turbulence. The first of these issues was examined by Carlson *et al.* (1984) in a detailed series of experiments testing the effects of various macroalgal and soil extracts on the growth of three of the ciguatera dinoflagellates. Growth of *G. toxicus* (in bacterized cultures) was enhanced by both soil extract and aqueous extracts of the macroalga *Chaetomorpha. Prorocentrum concavum* growth was stimulated by these same additions and by extracts of two other macroalgae, whereas *P. rhathymum* was inhibited by all such additions. Yasumoto *et al.* (1984) and Durand-Clement (1987) also report enhanced growth of *G. toxicus* following additions of soil extract, but other workers have reported inhibition of this species (Hurtel *et al.,* 1979).

One striking aspect of these results is that they are reminiscent of the state-of-knowledge about phytoplankton culture media prevailing 75 years ago (Pringsheim, 1912). In those days, phytoplankton growth in laboratory cultures was shown to require the addition of soil extract, ground-up copepods, or other poorly defined organics to the seawater base. It was subsequently shown by Provasoli *et al.* (1957) that the soil extract could be replaced by synthetic chelators (EDTA, NTA) and trace metals (iron, copper, zinc, manganese, cobalt, molybdenum). Progress in recent years has been even more dramatic, with chemically defined culture media being used to characterize the trace metal sensitivities (both toxic and nutritional) of a variety of phytoplankton species (reviewed by Huntsman and Sunda, 1980).

An hypothesis that follows from the above observations is that *G. toxicus* thrives in seawater that is oceanic rather than neritic in its chemical composition. One way to test this hypothesis would be to quantify the ciguatera dinoflagellates' sensitivities to, and requirements for, trace metals such as copper, zinc, manganese, and iron as has been done for other phytoplankton (Sunda and Guillard, 1976; Anderson and Morel, 1978; Brand *et al.,* 1983). Recent data indicate order of magnitude differences in zinc, manganese, and iron concentrations between oceanic and coastal waters (Bruland and Franks, 1983; Gordon *et al.,* 1982). Furthermore, Brand *et al.* (1983) showed that neritic species had significantly higher requirements for zinc and iron than oceanic species, which, when compared with measured concentrations of these trace elements in natural waters, suggested that the metals may be as important as nitrogen, phosphorus, and silicon in regulating marine ecosystems. In this context, note the recent demonstration by Entsch *et al.* (1983) that iron is a limiting nutrient for primary producers in Australian coral reefs.

The different responses of the ciguatera dinoflagellates to macroalgal and soil extract additions may be related to the spatial scale of their association with macroalgae. Both *G. toxicus* and *P. concavum* live attached to algal surfaces, suggesting a need for the organic substances and other nutrients commonly exuded by macroalgae (Steidinger, 1983). The growth of those two species was clearly stimulated by soil and macroalgal extracts. In contrast, *P. rhathymum* is most commonly reported to be free-swimming and thus may be adapted to water with different chemical characteristics than that immediately adjacent to macroalgae. *Prorocentrum rhathymum* growth was inhibited by macroalgal extracts and grew well in artificial seawater medium that could not support *G. toxicus* and *P. concavum* growth without additions of soil extract (Carlson *et al.,* 1984).

These reports of growth stimulation of *G. toxicus* by soil extract are unfortunately inconsistent with the general perception that this species does not thrive in areas subject to terrestrial runoff (Taylor, 1985). The beneficial effects of the poorly defined macroalgal extract additions to laboratory cultures described above may be indicative of a specialized nutritional interaction between the dinoflagellates and their host algae, but laboratory-prepared soil extract should be functionally similar to the material carried to coastal waters by terrestrial runoff. As suggested by Taylor (1986), it may be that the extraction and sterilization procedure used to obtain soil extract in the laboratory somehow alters its chemical characteristics and inactivates potentially toxic components. Whatever the reason, much work will be needed to define the trace metal and organic requirements of the ciguatera community if the apparent conflicts between field and laboratory observations are to be resolved. This is an appropriate time to apply established trace metal methodologies to the ciguatera dinoflagellates, since only through controlled manipulations of culture conditions will it be possible to identify the specific factors responsible for their variable growth characteristics and epiphytic life-style in natural waters.

One final series of laboratory culture studies deserves comment, having been initiated in part because of several observed positive and negative correlations between blooms of different species within the ciguatera dinoflagellate community. For example, Taylor and Gustavson (in press) commented on the inverse relationship between *G. toxicus* and *Ostreopsis* spp. blooms in the Caribbean, while Carlson (1984) found negative correlations between *G. toxicus* and both *P. rhathymum* and *A. carterae* abundance, and positive correlations between *P. rhathymum* and *P. concavum* in a major study in the Virgin Islands. In a subsequent series of laboratory experiments, Carlson (1984) demonstrated that *P. concavum* and *G. toxicus* produce ectocrines which inhibit each other's growth in bialgal culture. [*Gambierdiscus toxicus* actually produced a substance which was auto-inhibitory in batch cultures (Carlson, 1984).] Thus filtrates of *P. concavum* contained substances which were allelopathic to *G. toxicus* and stimulatory to *P. rhathymum,* but with little or no effect on re-inoculated *P. concavum* cells. This is similar to results from studies showing that ectocrines from other dinoflagellates can affect the growth of co-occurring diatoms and cyanobacteria (Pincemin, 1971; Uchida, 1981). Carlson speculated that the functional role of the ciguatera toxins may be to act as ectocrines which would enable a species to compete successfully with other epiphytic dinoflagellates and diatoms for space. Indeed, a tantalizing piece of preliminary data was recently presented which suggests that a maitotoxin fraction from *G. toxicus* prevented benthic diatoms from adhering to glass cover slips (D. G. Baden, reported in Hall and Shimizu, 1985).

## OVERVIEW

The general status of ecological research on the ciguatera dinoflagellates is that of a collection of observations and results that suggest certain relationships between the toxic dinoflagellates and their environment, their macroalgal hosts, and each other. These relationships are not documented thoroughly, however, and many contradictions or inconsistencies are apparent. This is true despite a long series of field studies by Yasumoto, Bagnis, and co-workers in the Pacific and extensive studies of the Virgin Islands by Carlson, Tindall, Taylor, and others. The incomplete nature of research into this phenomenon is in no way a reflection of the quality of the research by these workers. Indeed, their perseverance and methodical approach is to be commended. Instead, we must recognize that the causative organisms were first discovered only ten years ago, so field and laboratory methodologies are all relatively new. In addition, these organisms grow slowly in culture, they have unusual and varied requirements for culture medium, and they are both spatially and temporally patchy in the natural environment.

Considerably more effort has been invested in research into the chemical characteristics of the ciguatera toxins, but again the knowledge is incomplete. This is due to a different set of problems, the most important of which is the low concentration of the toxins in fish and in cultured dinoflagellates, making it difficult to obtain sufficient purified material for chemical analysis. Additional problems include the inconsistent and potentially incomplete chemical separation of the toxins from each other by different workers and the lack of a specific assay for each of the toxins.

Despite these limitations, much progress has been made and more is certainly forthcoming as ongoing work builds upon this preliminary base of knowledge. Certain areas of research seem especially important at this juncture. On the chemical side, there is a genuine need for the development of assay methodologies which will distinguish ciguatoxin, maitotoxin and okadaic acid from each other following a simple extraction procedure. Many are hopeful that the desired degree of specificity will come with the immunochemical assays that are now being developed. Concurrent with research into assay methodologies, standard extraction and purification procedures are needed so that the problems with impure preparations can be avoided. Once such procedures are established, it should then be possible to determine whether ciguatoxin is produced by *G. toxicus* (or other species) in laboratory culture and to study how that toxicity varies with growth conditions. If ciguatoxin production in culture can be verified and then maximized, the shortage of purified toxin that has limited progress so severely can rapidly be eliminated.

In addition to culture efforts directed at toxin production by the ciguatera dinoflagellates, considerable laboratory effort is needed to determine their nutritional requirements for, and sensitivities to, certain naturally occurring organic and inorganic compounds. If we knew why some dinoflagellate species choose to live attached to macroalgae, we might then have insights into the factors that regulate population abundance, especially those resulting in distinct seasonal cycles. Likewise, an understanding of the chemistry of the seawater surrounding the cells may lead to an appreciation of the factors that limit these species to areas free from terrestrial runoff. The production of ectocrines and other substances that affect co-occurring species is surely a fertile area for investigation and is one that may well explain certain population fluctuations. The time also seems right for the use of established techniques developed for the study of the effects of fluid flow on small organisms, with the eventual goal of learning the extent to which physics determines dinoflagellate/host preferences. In this case, and in virtually all of the above research directions, the more we move towards well-controlled laboratory cultures, flumes, and mesocosms, the better will be our quantitative understanding of these enigmatic organisms. Field studies are certainly of great value, but one of the lessons of the last decade has been that the natural habitat of the ciguatera dinoflagellates is complex and highly variable in both space and time and thus gives up its secrets very slowly.

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# STUDY OF PRODUCTION AND TOXICITY OF CULTURED *GAMBIERDISCUS TOXICUS*

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## ABSTRACT

*Gambierdiscus toxicus* has been described as the chief producer of ciguateric toxins: ciguatoxin (CTX) and maitotoxin (MTX). Dr. R. Bagnis (Malardé Institute, Tahiti) provided us with a strain of this benthic dinoflagellate in 1981 to study its cytological, physiological, and toxicological characteristics.

The growth of *G. toxicus* has been studied under various chemical and physical conditions enabling us to define optimal culture conditions. Since then, we have improved the cultivation procedure and have obtained large scale cultures of some clones and strains in different culture media.

All the experiments showed that *G. toxicus* has complex nutritive requirements and a large inertia to response at some non-drastic environmental variations. Increased growth rates were observed when cultures were treated with antibiotics.

The classical extraction procedure of the toxins has been adapted to our algal material. The method was simplified and resulted in enhanced toxinic yield.

Our principal results have demonstrated that *G. toxicus* in culture remained very toxic; 600 to 2000 cells were sufficient to kill a 20 g female mouse within 24 hours (MLD = 1–3 mg/kg). No variation of the degree of toxicity has been observed for three years.

A linear relationship between the number of *G. toxicus* cells (Coulter counted) and the weight of corresponding algal pellet has been found. This leads to an easier evaluation of the quantity of toxic extract required to calculate the minimum lethal dose (MLD).

The extraction procedure results in two toxic fractions: a water-soluble (MTX-like) and a lipid-soluble (CTX-like). The latter corresponds to 10 to 25% of the total toxicity.

A dose-time to death curve has been established with our CTX-like extracts.

## INTRODUCTION

Most tropical and intertropical coral reef seas contain the benthic dinoflagellate, *Gambierdiscus toxicus*. From wild *G. toxicus*, Yasumoto *et al.* (1977) extracted the two main toxins involved in ciguatera, ciguatoxin (CTX) and maitotoxin (MTX), which are transmitted to fish through the marine food chain (Taylor, 1979; Bagnis, 1981; Shimizu *et al.*, 1982; Withers, 1982). Although *G. toxicus* was the first dinoflagellate to be linked to the genesis of ciguateric toxins (Bagnis *et al.*, 1977), other toxic dinoflagellates, which are also potential sources of ciguateric toxins, have been isolated from ciguateric areas: *Prorocentrum lima* in the Pacific Ocean (Yasumoto *et al.*, 1984); and *Prorocentrum mexicanum* in the Atlantic Ocean (Tindall *et al.*, 1984). A number of dinoflagellates associated with the benthic community of coral reefs may contribute to the complex syndrome of ciguatera fish poisoning (Fukuyo, 1981;

Nakajima *et al.*, 1981; Steidinger and Baden, 1984), the biogenesis of which remains to be clarified.

We adapted a strain of *G. toxicus* from the Gambier Islands (kindly provided by Dr. R. Bagnis) to culture conditions in our laboratory and obtained mass cultures in order to study the main physiological, cytological, and ultrastructural characteristics of this dinoflagellate (Durand, 1984). We also established the toxicity of the culture under varying environmental conditions. The aim of this paper is to describe the main characteristics of cultured *G. toxicus.*

## CULTURE CONDITIONS AND PHYSIOLOGY OF *G. TOXICUS*

### *Temperature*

The temperature which supported optimum growth for our strains was 26 ± 1°C. Temperature over 30°C quickly killed the cells, and little growth was observed below 22°C. The temperature range for optimum growth was limited, probably due to the original collection site of the strains (the Gambier Islands), where little variation in seawater temperature occurs. Thus, our *G. toxicus* strains could be different from those found by Besada *et al.* (1982) in the Windley Keys (Florida), providing evidence of two distinct ecotypes.

However, after numerous transfers over one year, we obtained a strain of *G. toxicus* with satisfactory growth at 20°C.

### *Light*

By modifying light intensity and photoperiod we demonstrated that the growth rate of *G. toxicus* was largely influenced by the total amount of illumination received per day (Durand and Puiseux-Dao, 1985). The best conditions were a light intensity of 10 $W \cdot m^{-2}$ (tubes: Philips TL 65W and Mazda Fluor TF 65W) and a light/dark cycle of 10 h/14 h. Only a few divisions of *G. toxicus* were recorded under continuous light, suggesting that *G. toxicus,* like most dinoflagellates (Loeblich, 1966), has alternating light/dark dependence for division. Preliminary experiments showed that *G. toxicus* cultures can be synchronized by modifying the light/dark alternation as described for *Amphidinium carterae* (Galleron, 1976). The low light tolerance of *G. toxicus* could be related to its epiphytic habits (macroalgal substrates) in nature.

*G. toxicus* exhibited horizontal phototactic migrations at the bottom of the culture flasks in response to light intensity. Cells moved toward the light source in low-light conditions and moved in the opposite direction under high-light intensity. This behavior of *G. toxicus* in culture demonstrates its great sensitivity to light. We never observed eyespot or stigma structures with transmission optical studies.

### *Culture flasks, maintenance of the cultures, cell counting*

Clonal cultures were initiated by isolating a single cell under the microscope.

Stock cultures were maintained in 250 ml Erlenmeyer flasks. Experiments were conducted in 125 or 250 ml flasks (initial inoculate: 30–50 cells/ml) containing 70–80 and 150–170 ml of medium. For large cultures, *G. toxicus* was cultivated in 1 liter Fernbach flasks or 2 l Erlenmeyer flasks, since we were able to obtain a greater biomass production of this benthic dinoflagellate in these vessels than in large glass carboys. The cultures were harvested three or four weeks after the initial inoculation. At that time, *G. toxicus* cells overloaded the bottom of the flasks forming a unique algal

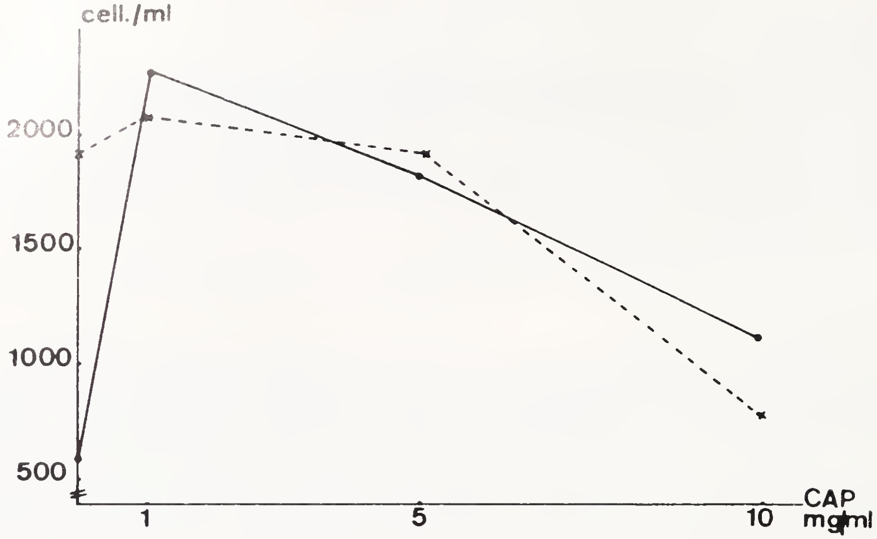


FIGURE 1. Effect of chloramphenicol (CAP) on *Gambierdiscus toxicus* growth in MPP. – – – Strain previously treated with CAP. —— Strain without previous CAP treatment.

layer. Cell density reached 4000 cells/ml (algal biomass equals 2 to $4 \cdot 10^6$ cells/1 liter Fernbach flask). Agitation of the cultures inhibited *G. toxicus* growth.

Just prior to stationary growth, cultures were harvested and washed by centrifugation (1500 × *g*, 5 min).

### *Influence of bacteria on* G. toxicus *growth*

Decreasing the bacterial flora improved the growth of *G. toxicus* cultures in our laboratory, from 500–1000 cells/ml final algal yield, to 4000 cells/ml. We used two methods for this purpose: serial washing in sterile medium and antibiotic treatment. To select the most efficient antibiotics, we tested the cultures with antibiograms (multidisk Sobioda 12GR422), then treated the cultures with either a large dose of antibiotics (5–20 mg/l) for a few hours in darkness (after which the drug was washed out), or with a smaller amount (0.1–1 mg/l) added directly to the culture medium. In the first case, bacterial spore germination was promoted by adding 0.1% neopeptone one or two days prior to treatment. The best results were obtained with rifampicine, minocycline, and chloramphenicol at 5 mg/l for 24 h. (See Fig. 1 for an example of the effect of chloramphenicol on *G. toxicus.*) The number of bacterial species was decreased, from more than 50 at the origin, to 3–5. We obtained a batch in which no bacteria could be detected during two months of growth. Presently, the use of chloramphenicol (5 mg/l) results in less than one bacterium per *G. toxicus* cell. Such cultures produce only a small amount of mucilage relative to contaminated cultures.

### *Culture media and nutrient supplementation assays*

Adjusting media to study the nutritional requirements of *G. toxicus* and to enhance its growth was difficult because of its slow response to environmental modifications. In addition, this large dinoflagellate showed a slow but efficient ability to adapt to various conditions.

We tried to cultivate *G. toxicus* in two different media, enriched seawater and artificial medium.

*Seawater media. G. toxicus* growth in seawater (from Banyuls/sur Mer, France) was slow (generation time: more than six days). For two months, the cells became progressively less pigmented and died.

In Lateur medium (Lateur, 1963: autoclaved seawater supplemented with $NaNO_3$, $Na_2HPO_4$, and soil extract), the growth remained slow (generation time: about five to six days) but the cultures did not degenerate.

One of the first media (Hurtel *et al.*, 1979) described for *G. toxicus* culturing was MPP, according to Provasoli and Pintner (Provasoli, 1958: autoclaved seawater supplemented with various mineral compounds and vitamins). In this medium, *G. toxicus* growth was satisfactory (division time: about three days). However, growth improved when we decreased the amount of enrichment solution (ES) from 2% to 1.5% and sometimes even 1%. When Tris (5 mg/l) was added, pH was adjusted to 8.15. No modification of *G. toxicus* growth was detected in cultures not supplemented with vitamins for six months.

*Artificial media.* The first cultures that we grew in ESAW medium (Harrison *et al.*, 1980) showed good growth for eight months and then degenerated suddenly, as reported by Carlson *et al.* (1984). Similar results ensued from a second attempt of cultures transferred in ESAW. Little amelioration of the algal growth could be obtained in this medium, even by removing NaF, silica, and SrCl or by the use of ES supplementation of MPP. Thus, ESAW is not suitable for our *G. toxicus* cultures.

In Shepard's artificial medium (MS: Shepard, 1969), *G. toxicus* growth was slow and irregular for many months (division time: five to eight days). No promotion of growth was obtained by modifying the salinity or the phosphorus concentration. In contrast, some growth stimulation occurred with the addition of 0.5% of ES (from MPP). At the moment, a strain that we progressively adapted to MS medium shows good growth (division time: four to five days) in this medium.

At present, large cultures of *G. toxicus* grow with a division time of two to four days in a mixture of MPP and MS (1:1).

*Nutrient supplementation assays.* For cultures grown in MPP medium, the following nutrient supplements did not result in a significant and reproducible improvement of culture yield: phosphates (0.1–1 m*M*), carbonates (0.5 m*M*), $Ca^{2+}$, $Mg^{2+}$, $K^+$ (0.1–1 m*M*), and glucose (1 g/l).

We tested various nitrogen sources to evaluate the nitrogen requirements of *G. toxicus*. The results are summarized below:

(1) *Mineral nitrogen:* a weak temporary increase of the division rate of *G. toxicus* was obtained by adding $NH_4Cl$ (below 0.5 m*M*) and $NaNO_3$ (5 m*M*), but these salts became toxic after a few days.

(2) *Organic nitrogen:* no amino acids tested (0.1 m*M*) stimulated *G. toxicus* growth. Addition of urea resulted in an increase of cell yield as shown in Figure 2: after one week, *G. toxicus* growth was enhanced by 0.5 m*M* urea, but this concentration became toxic; after three weeks, the largest biomass was obtained with 0.1 m*M* urea. We noticed that this stimulatory effect was more efficient while the cells were in active growth phase, suggesting that the algal metabolism must be high for urea to be used by *G. toxicus*.

(3) *Soil extract:* a variable but constant improvement of *G. toxicus* cultures was obtained by adding soil extract to MPP. We saw the following increases of the algal

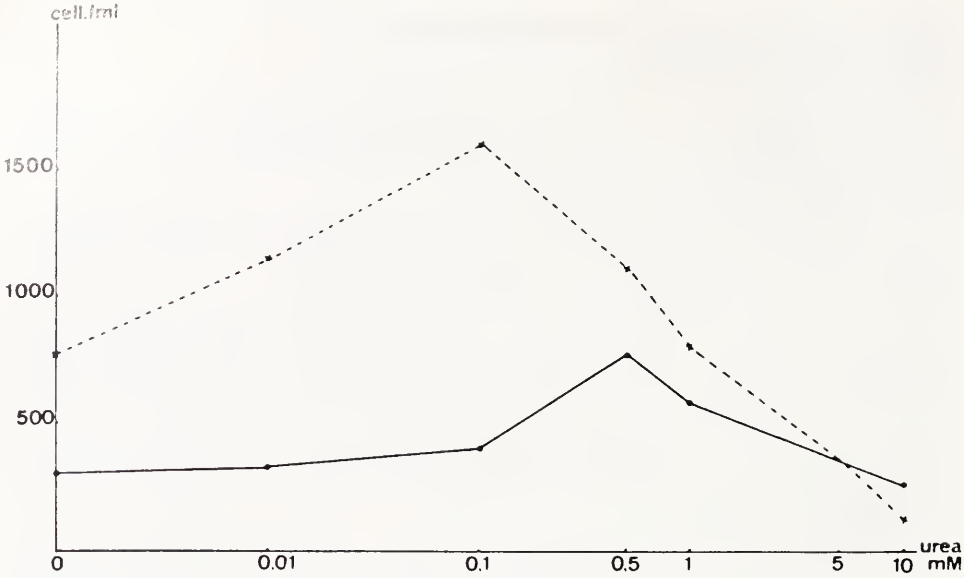


FIGURE 2. Effects of urea adjunction on *Gambierdiscus toxicus* growth in MPP medium. —— $t_0 + 7$ days. - - - $t_0 + 21$ days.

biomass 20 days after inoculation: ×1.8 for 1 g/l soil extract, ×2.4 for 2 g/l, ×2 for 4 g/l (toxic effect at higher doses). Other workers also obtained this stimulatory effect of soil extract (Carlson *et al.*, 1984; Yasumoto *et al.*, 1984).

(4) *Dilution of MPP:* diluting MPP with 5% distilled water stimulated *G. toxicus* growth (×1.5). This could be related to both a decrease of salinity and a dilution of toxic nutrients.

All the assays concerning the nutritional requirements for *G. toxicus* should be done again in MS medium (with the strain adapted to this medium) which has a well-defined chemical composition in contrast to seawater. *G. toxicus* possesses a complex physiology, and its study is complicated by the slow division rate and large size of this dinoflagellate, which allow slow responses to non-lethal variations of environmental parameters. In addition, analysis must consider the fact that cultured algae may present various adaptative behaviors, explaining the differences observed by different workers.

## PIGMENT COMPOSITION

Studies to determine the pigment composition of *G. toxicus* showed that this dinoflagellate contains unusual pigments (Durand and Berkaloff, 1985): it contains peridinin as the major carotenoid, and both chlorophylls $c_1$ and $c_2$. Peridinin is usually found only in association with chlorophyll $c_2$ (Jeffrey, 1976), and such pigment composition has been documented only in *Prorocentrum cassubicum.*

## MORPHOLOGICAL AND STRUCTURAL FEATURES OF *G. TOXICUS*

We observed cultured *G. toxicus* with both light and electron microscopes (transmission TEM and scanning SEM). A Zeiss standard WL microscope equipped with Nomarski optics was used for light microscopy.

For scanning electron microscopy, *G. toxicus* algal cultures were first pre-fixed by osmium vapors for 10 min, and then fixed by adding osmium (1% for 1 h at room temperature) to the culture medium. After centrifugation, the cell pellet was rinsed with distilled water, dehydrated in an acetone series, and dried using the $CO_2$ critical-point method. The cells were then coated with gold and examined in a Cambridge 600 SEM.

In the cultures, as a function of medium composition and light conditions, different morphological aspects of *G. toxicus* could be observed: typical armored cells, deformed cells, small motile cells and cysts.

### *Typical cells*

Figures 3a and b show typical *G. toxicus* cells. SEM observations were made to define accurately the thecal plate features and ornamentation of this dinoflagellate (Figs. 4a, b). Morphology and thecal plate organization were similar to that reported for wild cells by Adachi and Fukuyo (1979) and by Besada *et al.* (1982). The mean dorso-ventral diameter, for our cells, was 70 μm, with a range of 60–90 μm (30–50 μm for the vertical diameter). *G. toxicus* possesses the two typical flagella of dinoflagellates: a longitudinal one beating in the culture medium (about 50 μm length; see Fig. 3a) and a transversal one. The latter is located in the cingulum and has regular, jerky movements that substantially mixes the medium surrounding the cell.

Cell coloration varied, depending on light intensity and culture medium: *G. toxicus* cells appeared orange-brown in seawater, light brown in MPP, and dark brown in MS.

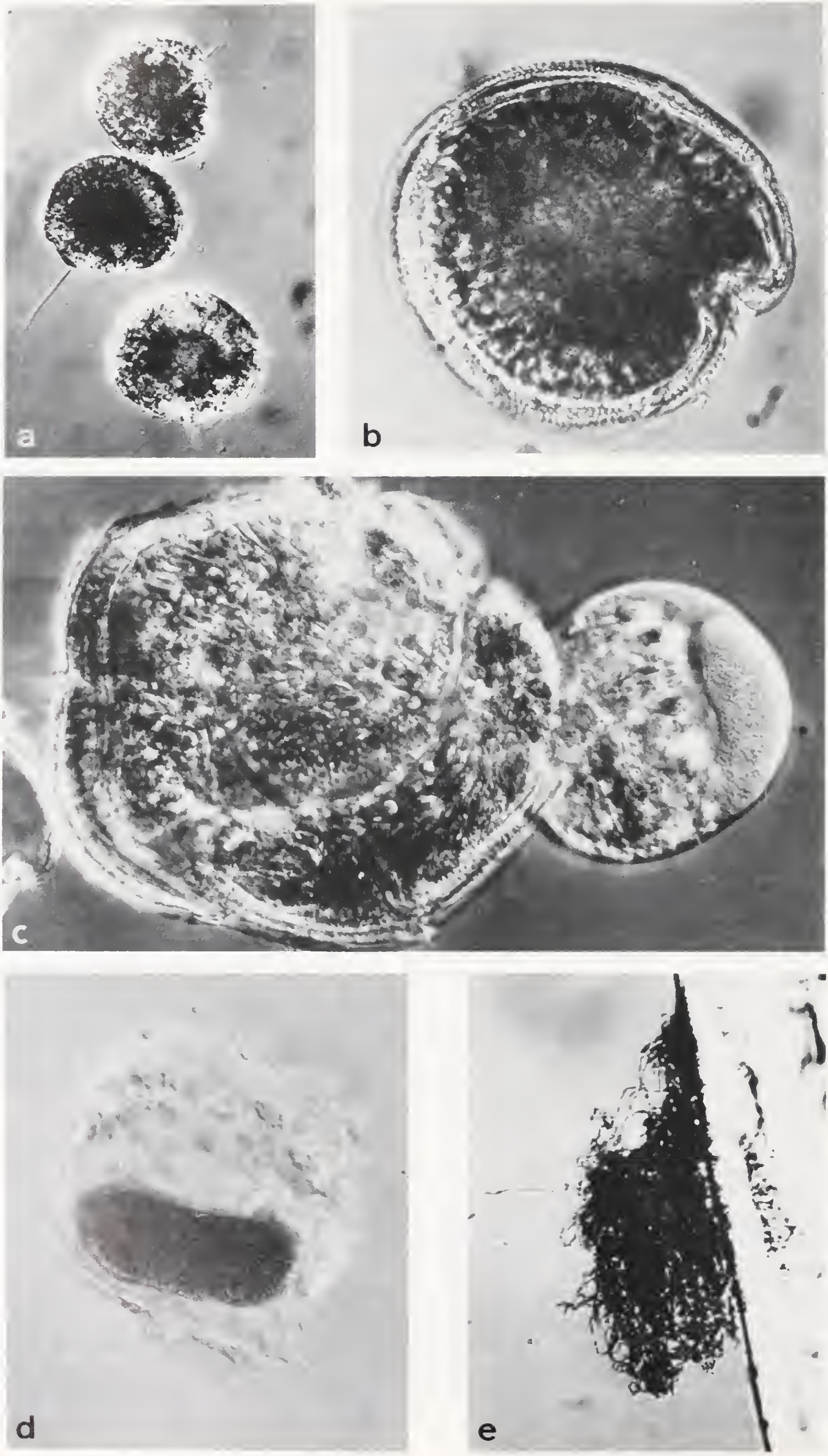
*Theca.* The *G. toxicus* cell covering consists of a thick theca (about 2 μm as seen in TEM), or amphiesma (Loeblich, 1970), which limits the cytoplasm. This covering is composed of an outer membrane, a well-developed plate layer, a dense pellicle, and a double membrane. The presence of the pellicle has been described by Morill and Loeblich (1981), who used chloral hydratehydriodic acid-iodine staining. This theca is perforated with numerous trichocystic pores.

*Nucleus.* The *G. toxicus* nucleus is large (30 μm; see Fig. 3d). It is easily ejected, intact, when the cell is pressed between a slide and a coverslip (Fig. 3c). It contains numerous permanently condensed chromosomes (Fig. 3e) and a large amount of DNA (162 pg/cell). Various nuclear stains revealed, in addition to this dinocaryon, numerous unusual structures that we called "pseudo-nuclear" vesicles: they possess nuclear features as shown by both cytochemical and electron microscopic studies (Durand *et al.,* 1986).

*Cytoplasmic features. G. toxicus* cytoplasm is very dense and contains the usual organelles found in dinoflagellates, including hundreds of trichocysts. When dehydrated (under the microscope, for instance), one or two brighter areas often appeared (Fig. 4f). They could be linked to the pusule.

### *Deformed cells*

Cells having abnormal morphological aspects appeared in the cultures under some conditions (Durand and Puiseux-Dao, 1985). The morphology of these cells was deformed in comparison to typical cells: in apical view, their shape was embossed instead of circular; in a lateral view, they were round instead of flattened. The thecal-plate feature of teratogenous forms was completely disorganized as seen by SEM (Fig. 4c). Their size was large (80–100 μm), their motility reduced, and they did not stick to the bottom of the flasks. They were capable of mitosis, so they cannot be a sexual stage of *G. toxicus.*


a
b
c
d
e

Analysis of the conditions of the appearance and evolution of deformed cells in *G. toxicus* populations suggested two sources: culture-medium composition and photoperiod.

### *Small motile cells and cysts*

Cells that had abandoned their theca (ecdysis; Fig. 4d) were often observed. Some small, motile, and apparently naked cells were occasionally visible in aged cultures (Fig. 4e). They could correspond to the cells released by ecdysis described above and be a survival behavior as described for other dinoflagellates (Walker, 1982), or to a stage of hypnoid cyst formation defined by Anderson *et al.* (1978). According to these workers, the structure shown in Figures 4g and h (same cell, two different foci) would represent another type of temporary cyst (coccoid). They were detected only once, after increasing the temperature of the culture to over 30°C.

Some large (over 100 $\mu$m), dark, and non-motile cells which could be interpreted as hypnocysts, were occasionally observed. Also observed (mostly in seawater) were paired cells which could be interpreted as fused gametes. These observations suggest that sexual reproduction might occur in *G. toxicus* cultures, but this hypothesis needs to be confirmed by other observations and studies.

## TOXICITY OF *G. TOXICUS* CULTURES

*G. toxicus* toxicity was studied to evaluate the influence of culturing on its ability to synthetize toxic compounds. After verifying that *G. toxicus* was toxic, we tried to improve the toxin extraction procedure; finally, we tested a few parameters which could modify the toxin production of this dinoflagellate.

### *Evaluation of toxicity*

Toxicity was evaluated using the mouse bioassay described by Yasumoto *et al.* (1979). Toxic extracts were dried under a nitrogen stream to eliminate any trace of solvent. Each extract was emulsified in 0.4 ml of a Tween 60 solution and injected intraperitoneally into a 19–21 g mouse (Swiss female). Several doses were tested (2 mice per dose) to determine the minimal lethal dose (MLD). MLD is defined as the minimal amount of extract that causes death for a 20 g mouse within 24 h (expressed in mg/kg). To simplify comparison between the toxicity of different extracts, lethality is expressed in mouse-units (one MU equals MLD ÷ 50), and total lethality is expressed as the number of cells per mouse unit.

### *Toxin extraction procedure*

First, we extracted ciguatoxins by the classic procedure of Yasumoto *et al.* (1979) and Bagnis *et al.* (1980). This procedure was inadequate to our algal material, so we studied the yield of each step of the protocol and modified them progressively. These

FIGURE 3. *Gambierdiscus toxicus* cells. (a) Three typical *G. toxicus* cells (microcinematography, see the longitudinal flagellum), 320×. (b) A typical *G. toxicus* cell, 900×. (c) *G. toxicus* nucleus (Nu) ejected with the cytoplasm while pressing the cell between slide and coverslip, 1150×. (d) A depigmented *G. toxicus* cell after Feulgen's staining, 800×. (e) Squash of *G. toxicus* after Feulgen's staining: view of the chromosomes, 2600×.

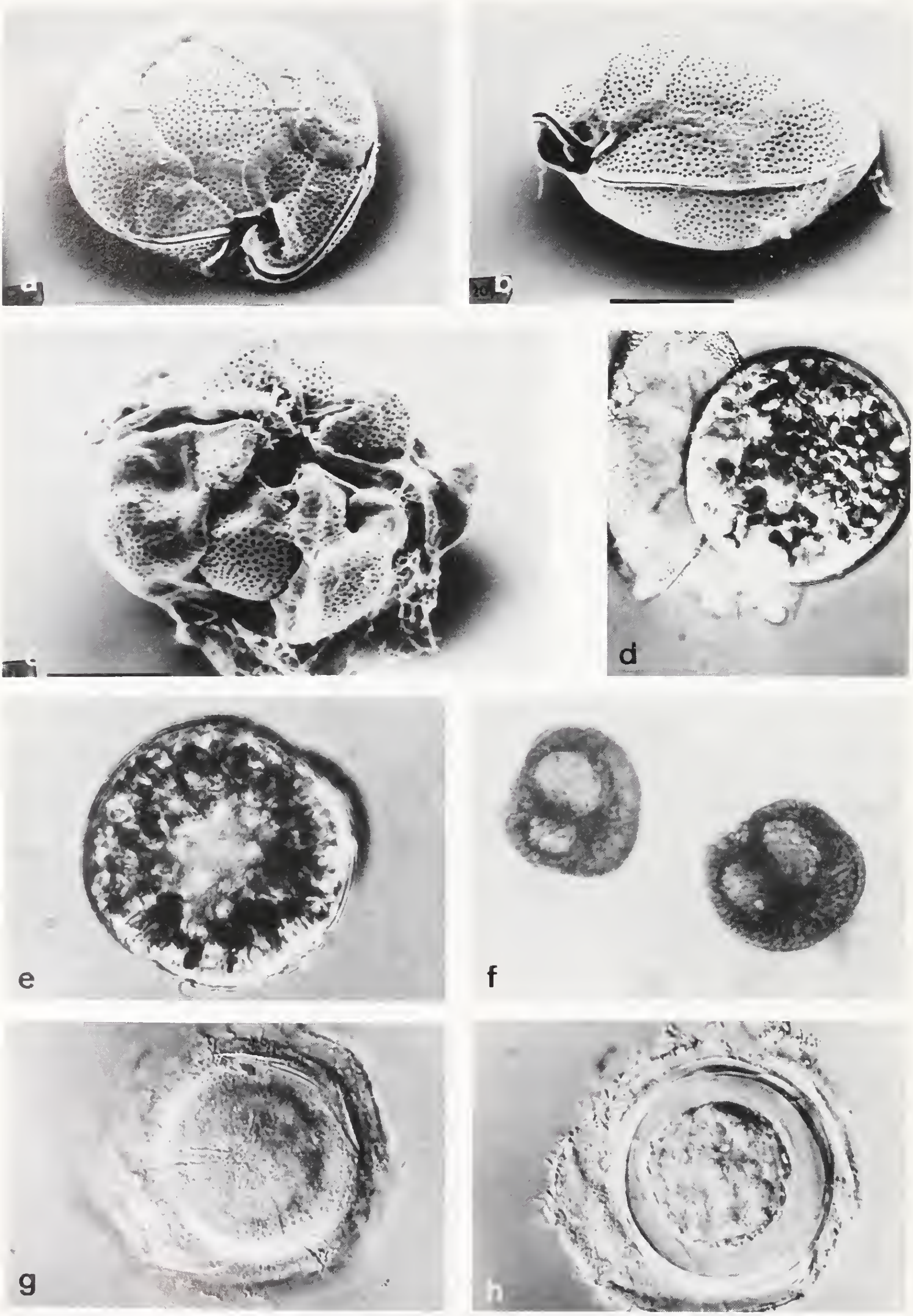


FIGURE 4. (a, b) Typical *G. toxicus* cells, SEM. The black line represents 20 μm (Durand and Coute photographs). (c) A deformed *G. toxicus* cell, SEM. The black line represents 20 μm. (d) Cell during ecdysis, 600×. (e) Aspect of a small, apparently naked cell, 900×. (f) Two typical cells after small dehydration process, 350×. (g, h) Aspect of a "temporary cyst" of *G. toxicus* (2 different foci), 600×.

TABLE I

*Toxin extraction procedure for* Gambierdiscus toxicus *cultures*

*G. toxicus* culture (about $10^6$ cells)

⇩

Centrifugation ($1500 \times g$—5 min)

⇩

Sonication of the pellet in methanol

⇩

Extraction of the pellet by methanol
(10 ml, ×3, room temperature)
TOTAL CRUDE EXTRACT

⇩

Evaporation of methanol

⇩

Partition: water pH 7/isopropylic ether
(2 V) ↙ ↘ (1 V)
AQUEOUS-PHASE ETHER-PHASE

assays resulted in the protocol outlined in Table I. The principal modifications were: (1) using centrifugation, instead of filtration on paper filters, for culture harvesting and cell sonication, which permitted us to use small amounts of solvent; and (2) extracting with "cold" (room temperature) methanol and partitioning with isopropylic ether. This method is simpler, more rapid, and enhanced the yield of toxin two- to fourfold.

To simplify the evaluation of MLD for the initial methanol extract and to save mice, we established a relationship between the cell-pellet weight and the corresponding cell number (calculated using a Coulter counter). The equation of the linear relationship is:

$$Y = 0.056 + 0.263X$$

(Y represents the cell number: X represents the weight; n = 48 samples tested).

Thus, we established that one million *G. toxicus* cells weighed 0.36 g.

### *Results*

For toxicity screening we extracted many batches of *G. toxicus* cultivated in different media conditions. We also looked for differences between clonal and non-clonal strains. For these experiments cultures were harvested when they reached the stationary growth phase. Results are given in Table II.

Analysis of the data shows that differences between toxicities for various culture conditions were not significant. Other preliminary assays suggest that bacteria have little influence on total toxin production of *G. toxicus,* as the same toxicities were found for contaminated cultures (prior to antibiotic treatment) and "axenized" cultures (less than one bacteria per dinoflagellate cell) (Durand, 1987). In addition, we did not find a large increase in toxicity in aged cultures, in contrast to what other workers reported (Yasumoto, *et al.,* 1979; Bergmann and Alam, 1981).

TABLE II

*Compilation of* Gambierdiscus toxicus *toxicity data*

| Strain | Culture medium | Cell number ($10^3$) | Total crude extract | | Toxicity % | |
|---|---|---|---|---|---|---|
| | | | Total MU | Cells/MU | Ether-phase | Aqueous-phase |
| Clone 1 | MPP | 539 | 800 | 680 | ND | ND |
| | MS + MPP | 324 | 625 | 520 | 8 | 72 |
| | | 288 | 290 | 1000 | 34 | ND |
| Clone 2 | MS + MPP | 550 | 550 | 1000 | 18 | 82 |
| | | 218 | 180 | 1220 | 3 | ND |
| | LATEUR | 421 | 190 | 2240 | 8 | ND |
| | | 1843 | 1860 | 980 | ND | ND |
| Clone 3 | MPP | 307 | 340 | 900 | 3 | 56 |
| | | 205 | 180 | 1140 | 4 | ND |
| | MS | 203 | 150 | 1360 | 10 | 83 |
| | MS + MPP | 1500 | 1700 | 880 | 12 | 88 |
| | | 769 | 750 | 1020 | ND | ND |
| Clone 4 | MS + MPP | 243 | 235 | 1040 | 11 | 60 |
| | | 1640 | 700 | 2340 | 28 | 71 |
| Clone 5 | MPP | 326 | 400 | 820 | ND | ND |
| | | 400 | 625 | 640 | 19 | 74 |
| | MS | 336 | 225 | 1500 | ND | ND |
| | MS + MPP | 1040 | 420 | 2480 | ND | ND |
| | | 166 | 170 | 980 | ND | ND |
| Strain A | MPP | 726 | 480 | 1520 | ND | ND |
| | MS + MPP | 568 | 470 | 1200 | 13 | 74 |
| | | 2200 | 1050 | 2100 | 18 | 81 |
| Strain B | MPP | 1486 | 1560 | 960 | 13 | 32 |
| | | 2015 | 4075 | 500 | 21 | 54 |
| | MS + MPP | 727 | 725 | 1000 | ND | ND |

ND: non-determined; MU: mouse unit [equals Minimal Lethal Dose (MLD) ÷ 50]; MPP: culturing medium (Provasoli, 1958); MS: Shepard's artificial medium (Shepard, 1968).

The mean lethality for our *G. toxicus* strains was 1200 cells/MU.

$$10^6 \text{ } G.\ toxicus = 0.36 \text{ g} = 800 \text{ MU}; \qquad \text{MLD} = 1\text{–}3 \text{ mg/kg}.$$

We concluded that, under laboratory culture conditions, *G. toxicus* remained very toxic—in the same range that was reported for wild populations (taking into account the extraction yield by Yasumoto *et al.,* 1979, and Bagnis *et al.,* 1980).

## *CTX-like and MTX-like toxicities*

During the toxin extraction procedure, the partition step always resulted in two toxic fractions: a water-soluble fraction (MTX-like) and an ether-soluble fraction (CTX-like). These fractions appeared in a CTX-like/MTX-like ratio of about 0.2. Figure 5 gives the dose/time-to-death curve that was obtained with one CTX-like extract. It must be noted that the minimal death time for this toxic fraction is higher than that reported for ciguatoxin extracted from fish (Tachibana, 1980; Lewis and Endean, 1984). This suggests that the ether phase obtained from *G. toxicus* cultures

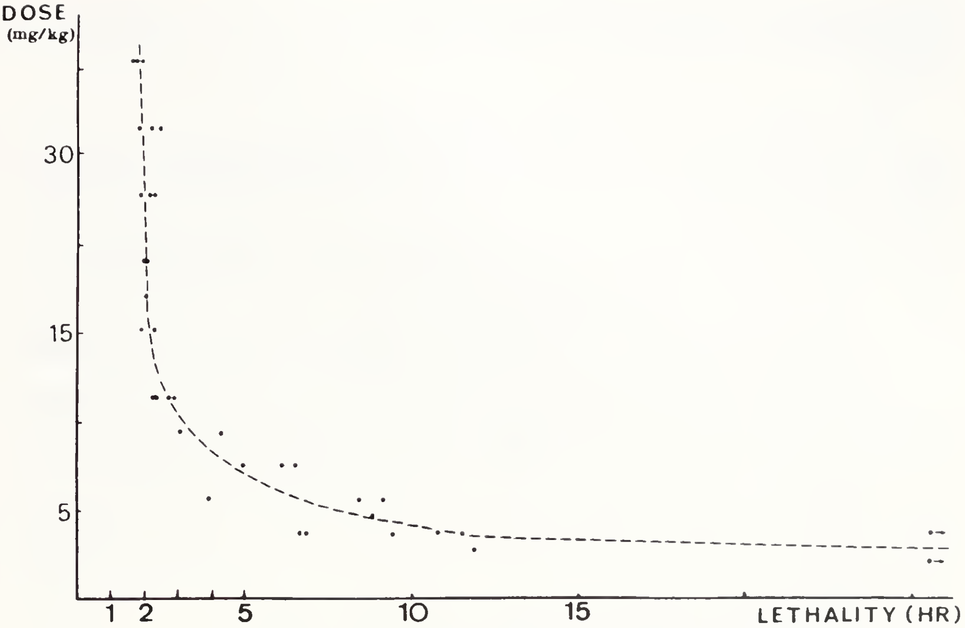


FIGURE 5. Relationship between dose and death-time for a crude ether phase from *Gambierdiscus toxicus* cultures (intraperitoneal injection in 20 g mice).

is not equivalent to the one obtained from fish. In particular, it could contain MTX (as already suggested by Yasumoto *et al.*, 1979), CTX-MTX complex, or another toxic compound. Further studies are necessary to characterize the toxin content of *G. toxicus*.

## CONCLUSION

We obtained some stable, well-defined uni-algal cultures of *G. toxicus* (different clones, in different media). Development of large-scale culturing must, at present, take into account the benthic behavior of this dinoflagellate, and should employ similar techniques as those used for animal-cell cultures.

*G. toxicus* in culture remains very toxic, thus it could be a superior raw material for providing cleaner and more abundant amounts of toxins: certainly maitotoxin and perhaps ciguatoxin, if further toxin characterization confirms its presence in the ether phase. The relationship between the two toxins remains to be elucidated, as does the importance of *G. toxicus* in ciguatera outbreaks. As this dinoflagellate is the focus of several studies, we suggest a standardization of its toxicity evaluation in order to help comparisons.

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# RECENT DEVELOPMENTS IN THE MOLECULAR STRUCTURE OF CIGUATOXIN

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## ABSTRACT

Crystalline ciguatoxin isolated from moray eel (*Lycodontis* ≡ *Gymnothorax javanicus*) viscera has an $LD_{50}$ of 0.45 g/kg (i.p., mice). It has a molecular weight of 1111.7 ± 0.2 daltons. $^1H$ NMR studies have shown that it is a polar and highly oxygenated molecule belonging to the class of polyethers. On basic alumina ciguatoxin is reversibly converted to a chromatographically distinct less polar form, which is equally toxic and elicits typical ciguatoxin symptoms in mice.

From parrotfish (*Scarus sordidus*), which originated on a ciguateric reef on Tarawa atoll (Kiribati), we have isolated two toxins that evoke ciguatera symptoms in mice at approximately equal levels. Chromatographic evidence suggests that the two toxins are identical with the two ciguatoxins of different polarity and that the less polar form is the previously described scaritoxin.

## INTRODUCTION

> "He who oversees everything also created very many poisonous fish, in this way he punishes those who seek them."
>
> —*J. Grevin*

In his book on venoms the 16th century, French physician and poet Jacques Grevin (Grevin, 1568) intuitively foresaw some of the frustration which has been the hallmark of ciguatera research during the past thirty years. Although ciguatoxin, the principal toxin in ciguateric carnivorous fish, may be superficially compared with the well-known marine toxins tetrodotoxin and the saxi-gonyautoxins, they share few characteristics with ciguatoxin. The single factor responsible for the slow progress in ciguatera research is that ciguatoxin is a slow-acting toxin which is rarely fatal to man. It is rarely fatal not because of its lack of potency—indeed its potency is surpassed only by that of palytoxin (0.15 $\mu$g/kg; Moore and Scheuer, 1971) and maitotoxin (0.2 $\mu$g/kg; Ohizumi and Yasumoto, 1983)—consumers of ciguateric fish survive because of its extremely low concentration in fish flesh. Thus, it has been difficult to accumulate enough toxin for structural research and—for many years—to convince skeptical fellow scientists of the very existence of a well-defined toxic entity. As a slowly acting toxin, ciguatoxin lacks the dramatic impact of a fast-acting toxin, somehow a convincing demonstration of the power of a toxin.

## METHODS AND RESULTS

Because of the many variables attending ciguatera outbreaks, among them place, time, species of fish, and reported symptoms in man, we have confined our research to toxin isolated from the moray eel (*Lycodontis* ≡ *Gymnothorax javanicus*), initially from flesh and viscera, but more recently exclusively from viscera in order to economize on solvents and shipping cost. Geographically, the eels originated from John-

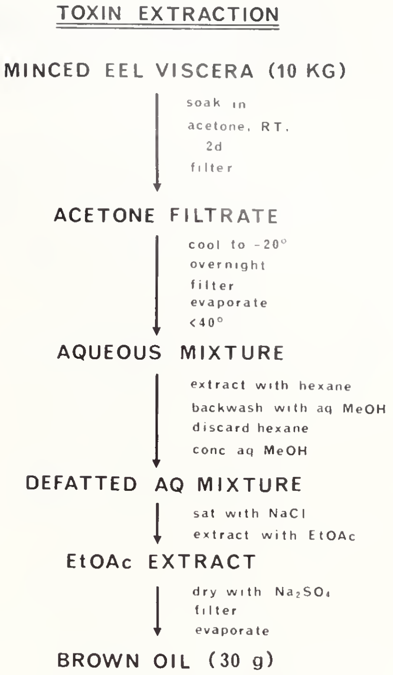

TOXIN EXTRACTION
MINCED EEL VISCERA (10 KG)
soak in acetone, RT, 2d
filter
ACETONE FILTRATE
cool to -20°
overnight
filter
evaporate
<40°
AQUEOUS MIXTURE
extract with hexane
backwash with aq MeOH
discard hexane
conc aq MeOH
DEFATTED AQ MIXTURE
sat with NaCl
extract with EtOAc
EtOAc EXTRACT
dry with Na2SO4
filter
evaporate
BROWN OIL (30 g)

FIGURE 1. Ciguatoxin extraction.

ston atoll (165°W, 17°N) and more recently from Tarawa atoll (173°E, 1°30'N), Republic of Kiribati.

Figures 1 and 2 summarize our procedure for toxin extraction and purification (Tachibana, 1980). Approximately 75 kg of eel viscera, representing some 1100 kg of toxic eels, yielded 1.3 mg of chromatographically pure toxin. The final HPLC trace (Fig. 3) obtained on a $C_{18}$ reversed phase silica column is a symmetrical peak. The toxin, $LD_{50}$ 0.45 $\mu$g/kg (i.p., mice), is a colorless solid readily soluble in methanol, ethanol, 2-propanol, and acetone, sparingly soluble in chloroform and diethyl ether, and nearly insoluble in water or benzene.

Ciguatoxin displays a single UV absorption peak at 215 nm ($\epsilon$ 5250). At that wavelength its CD (circular dichroism) molecular ellipticity is −620. The most prominent features in the infrared spectrum (FT, solid film) of ciguatoxin are hydroxyl (3450 $cm^{-1}$) and ether (1080 $cm^{-1}$) bands. A respectable signal at 1600 $cm^{-1}$ cannot be unequivocally interpreted.

Californium-252 plasma desorption mass spectrometry pointed to a likely molecular weight of 1111.7 ± 0.3 daltons (R. D. Macfarlane and C. McNeil, pers. comm.). Since this technique is unsuitable for high resolution measurements, no molecular formula of ciguatoxin was obtained. On the basis of $^1$H NMR data, formulas such as $C_{53}H_{77}NO_{24}$ (1112.2) or $C_{54}H_{78}O_{24}$ (1111.2) are reasonable.

The bulk of the structural information was derived from extensive $^1$H NMR experiments at 360 and 600 MHz in methanol-$d_4$ or dimethylsulfoxide-$d_6$. Methanol gives rise to a well-defined spectrum, but provides no clues for exchangeable protons. Ciguatoxin possesses five hydroxyl groups, four carbon-carbon double bonds, and five methyls, all on saturated carbon. The combined structural pieces account for

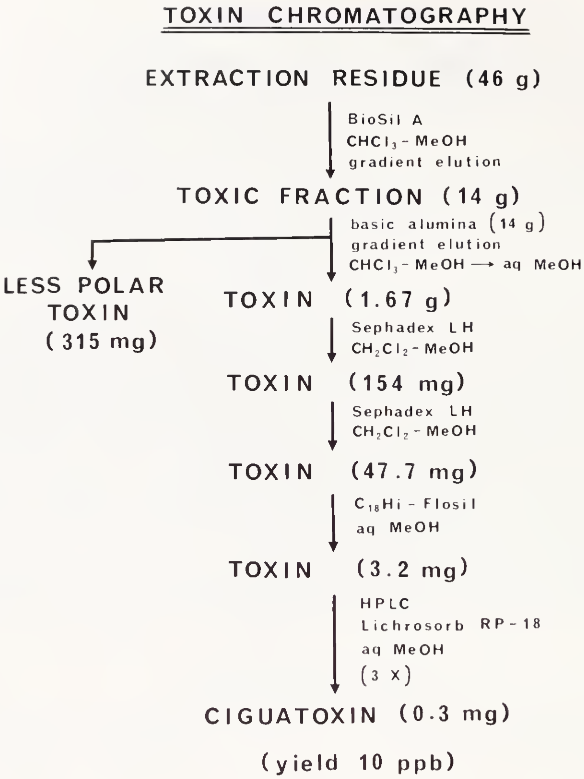

TOXIN CHROMATOGRAPHY
EXTRACTION RESIDUE (46 g)
BioSil A
CHCl₃ - MeOH
gradient elution
TOXIC FRACTION (14 g)
basic alumina (14 g)
gradient elution
CHCl₃ - MeOH ⟶ aq MeOH
LESS POLAR TOXIN (315 mg)
TOXIN (1.67 g)
Sephadex LH
CH₂Cl₂ - MeOH
TOXIN (154 mg)
Sephadex LH
CH₂Cl₂ - MeOH
TOXIN (47.7 mg)
C₁₈Hi - Flosil
aq MeOH
TOXIN (3.2 mg)
HPLC
Lichrosorb RP - 18
aq MeOH
(3 x)
CIGUATOXIN (0.3 mg)
(yield 10 ppb)

FIGURE 2. Ciguatoxin purification.

nearly 75 hydrogen atoms and imply 54 carbon atoms, but in the absence of a carbon spectrum it is impossible to judge the degree of overlap, which casts doubt on the reliability of the carbon count. Although no satisfactory carbon-13 spectrum of ciguatoxin has been determined because of lack of toxin and/or sufficient instrument time, our sample crystallized in an NMR tube during an attempt to have the carbon spectrum measured at a mainland applications laboratory. The crystals, unfortunately, are too small to be suitable for x-ray diffraction studies, and our attempts at growing larger crystals have so far failed.

## DISCUSSION AND CONCLUSION

When one considers the broad spectrum of symptoms which ciguatera-intoxicated patients describe and the difficulty of isolating a homogeneous toxin from a complex matrix in which it is present at a concentration of approximately $1 \times 10^{-6}\%$, the question of multiple toxins inevitably arises. In the absence of convincing evidence to the contrary, we have followed the simplest assumption in our research; *i.e.,* we have assumed that ciguatoxin is a single entity. Yet occasionally we observed (Tachibana, 1980) that in samples of extracts that had been stored for some time, ciguatoxin would be eluted from a silica column with a less polar solvent mixture (chloroform/methanol 97:3) than is the case normally, when the bulk of the toxin is eluted with a 9:1 chloroform/methanol solution. The existence of a less polar form

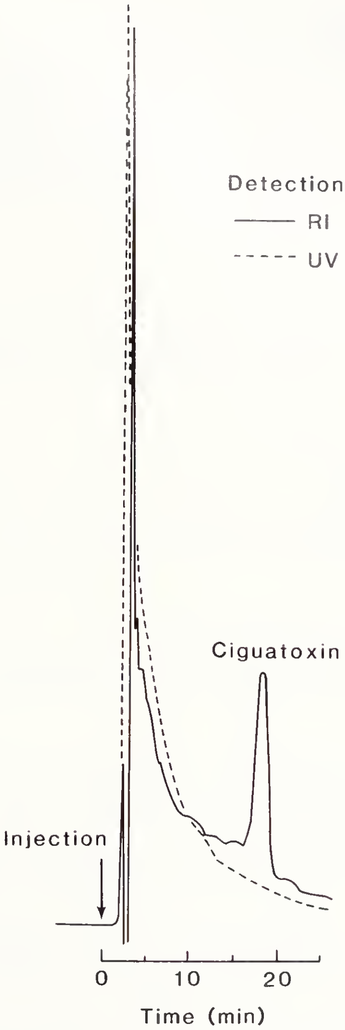
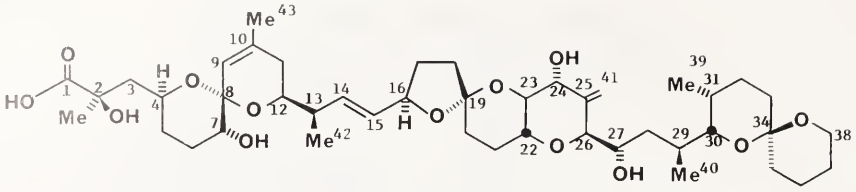


FIGURE 3. Reversed phase HPLC of ciguatoxin.

of ciguatoxin can be demonstrated by chromatography on basic alumina of different activity grades. We showed (Nukina *et al.*, 1984) that the two forms of ciguatoxin, while chromatographically distinct, are interconvertible. The two forms have $^1$H NMR spectra which differ only in minor details and elicit comparable symptoms in mice.

An epidemiological survey including detailed case studies in the Gambier islands, where ciguatera intoxication arises principally from eating parrotfishes (Scaridae), led Bagnis *et al.* (1974) to suggest that either a new toxin or multiple toxins were involved. In her follow-up, Chungue (1977; Chungue *et al.*, 1977) isolated from the flesh of *Scarus gibbus* a toxic mixture which was separable by DEAE cellulose chromatography into a toxin designated scaritoxin and a more polar toxin which strongly resem-



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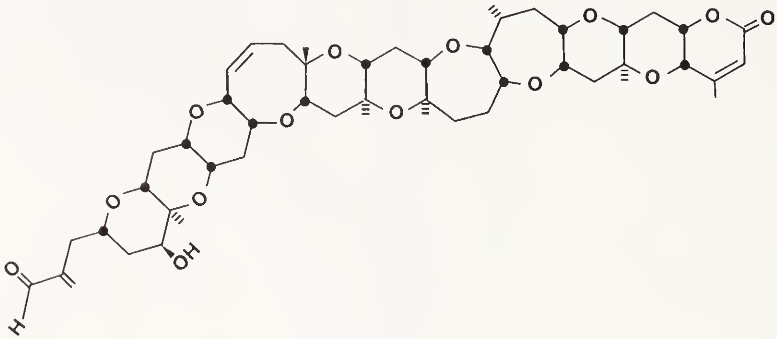

2

FIGURE 4. (1) Molecular structure of okadaic acid. (2) Molecular structure of brevetoxin B.

bled ciguatoxin. Scaritoxin was reported to cause severe hind limb paralysis in mice, a symptom not normally observed with ciguatoxin.

Recently, we (Joh and Scheuer, in press) examined parrotfish (*Scarus sordidus*) from a toxic reef on Tarawa atoll, Republic of Kiribati. We also isolated two toxins separable on DEAE cellulose. However, by manipulation on basic alumina we were able to interconvert the two toxins. By TLC comparison we showed that scaritoxin and the less polar ciguatoxin (Nukina *et al.*, 1984) are identical, though this finding remains to be confirmed by spectral comparison.

When Yasumoto (Murakami *et al.*, 1982) examined the constituents of the toxic dinoflagellate *Prorocentrum lima,* he made the surprising discovery that on TLC analysis one of the constituents was indistinguishable from ciguatoxin. The substance proved not to be ciguatoxin but okadaic acid (Fig. 4, 1), a compound which we had shortly before reported from a sponge, *Halichondria okadai* (Tachibana *et al.*, 1981). The two compounds differ greatly in size (1111 *vs.* 804 daltons) and lethality (0.45 *vs.* 192 $\mu$g/kg), but evidently not in polarity because of their similar chromatographic behavior. This was the first clear indication that ciguatoxin belongs to the class of polyethers, as does, *inter alia,* brevetoxin B (Fig. 4, 2) (Lin *et al.*, 1981). These compounds are highly oxygenated long-chain fatty acids, in which most of the oxygen atoms occur as cyclic ether linkages. Okadaic acid (Fig. 4, 1), possesses one carboxyl, four hydroxyls, and seven oxa rings, in addition to three carbon-carbon double bonds. This information allows us to extrapolate safely to the ciguatoxin structure, which evidently is a complex polyether possessing five hydroxyls and four carbon-carbon double bonds. This close structural analogy to okadaic acid (Fig. 4, 1) and to brevetoxin (Fig. 4, 2) provides a plausible rationale for the interchangeability of different chromatographic forms of ciguatoxin and scaritoxin. The presence in ciguatoxin of multiple hydroxyl groups permits formation and destruction of various hydrogen-bonded forms while preserving the structural integrity of the molecule. Because of its large size, ciguatoxin may well assume two or more secondary shapes which prevail under different conditions of basicity.

Perhaps the most intriguing questions posed upon examination of these compounds are those dealing with their mechanism of physiological action and with the subtle structural features that give rise to profound differences in lethality and overt symptoms in mammals.

## ACKNOWLEDGMENT

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# TOXINS PRODUCED BY BENTHIC DINOFLAGELLATES

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## ABSTRACT

Nine species of benthic dinoflagellates collected in subtropical waters were cultured, extracted, and tested for mouse lethality, ichthyotoxicity, and hemolytic activity. Hemolytic activity was detectable in all species, but the activities of *Amphidinium carteri, A. klebsi,* and *Gambierdiscus toxicus* were outstanding. *G. toxicus* showed the most potent mouse lethality. Two hemolytic constituents of *A. carteri* were determined to be mono- and di-galactoglycerolipids. Maitotoxin, produced by *G. toxicus,* was suggested to have a molecular weight of 3402 ± 2 ($m/z$). Two potent toxins against mice were isolated from *Prorocentrum lima* and identified as okadaic acid and 5-methylene-6-hydroxy-2-hexen-1-okadaate.

## INTRODUCTION

Scientists first noticed the toxigenicity of benthic dinoflagellates when *Gambierdiscus toxicus* was found to produce and to transmit ciguatoxin and maitotoxin to herbivorous fish (Yasumoto *et al.,* 1977). Subsequent *G. toxicus* distribution surveys revealed an abundance, in terms of both species and population, of benthic dinoflagellates in coral reef communities. This observation suggests that toxic metabolites, if any, of these benthic species are taken up by herbivorous fish, as ciguatoxin is, and contribute to the manifestation of the complex symptoms of ciguatera. The actual occurrence of minor toxins in the viscera of herbivorous fish has been confirmed, and the toxigenicity of several benthic species has also been demonstrated previously (Yasumoto *et al.,* 1976; Nakajima *et al.,* 1981). The present paper briefly summarizes our knowledge of the toxic benthic species and the chemical natures of their toxins; ciguatoxin, however, has been described separately by Tachibana *et al.* (1986).

## MATERIALS AND METHODS

Benthic dinoflagellates collected at Okinawa, Japan, were cultured in a nutrient-enriched seawater medium described by Provasoli (1968). The following nine species were tested for the toxin production: *Amphidinium carteri, A. klebsi, Coolia monotis, Gambierdiscus toxicus, Ostreopsis ovata, O. siamensis, Prorocentrum concavum, P. lima,* and *P. rhathymum.*

The harvested cells were extracted with boiling methanol. The methanol was removed by evaporation, and the residue was then suspended in water and extracted, first with diethyl ether and then with 1-butanol. The residues obtained after evapora-

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TABLE I

*Toxicigenicity of benthic dinoflagellates*

| Species | Mouse lethality | Ichthyotoxicity | Hemolytic activity |
|---|---|---|---|
| *Amphidinium carteri* | + | ++ | ++ |
| *Amphidinium klebsi* | ++ | ++ | ++++ |
| *Coolia monotis* | − | − | + |
| *Gambierdiscus toxicus* | +++++ | − | +++++ |
| *Ostreopsis ovata* | + | − | + |
| *Ostreopsis siamensis* | +++ | − | + |
| *Prorocentrum concavum* | ++ | +++ | + |
| *Prorocentrum lima* | +++ | − | + |
| *Prorocentrum rhathymum* | − | − | + |

The relative potency is expressed by increasing the number of +, the potency of undetectable level is expressed by −.

tion of the solvents were tested, respectively, for mouse lethality, ichthyotoxicity, and hemolytic activity, as described previously (Nakajima *et al.*, 1981).

Three toxins, tentatively named PL toxin-1, -2, and -3, were isolated from *P. lima* by successive treatments on columns of silicic acid ($CHCl_3$-MeOH, stepwise), Sephadex LH-20 ($CHCl_3$-MeOH 2:1), LiChroprep RP-2 (Merck, MeOH-$H_2O$ 2:1), and ODS (Kyowaseimitsu, MeOH-$H_2O$ 4:1).

Five hemolytic compounds (hemolysin-1 to -5) were present in *A. carteri*. Hemolysin-1 and -2 were purified on columns of silicic acid ($CHCl_3$-MeOH, stepwise) and ODS Q-3 (Fujigel, MeOH-$H_2O$ 9:1). Hemolysin-3 to -5 were also purified in a similar manner but further purification on a Toyopearl 40 column was necessary (MeOH-$H_2O$, stepwise).

Purification of maitotoxin was carried out on columns of silicic acid ($CHCl_3$-MeOH, stepwise), Develosil ODS (Nomurakagaku, MeOH-$H_2O$, stepwise), and Develosil TMS (MeCN-$H_2O$ 35:65).

$^1H$ NMR and $^{13}C$ NMR spectra were taken on either a Nicolet NT 360 spectrometer or a JEOL FX-100 spectrometer, and mass spectra were taken on a Hitachi M-80 mass spectrometer.

## RESULTS

Bioassays indicate that all the dinoflagellates are toxic by at least one of the assay methods, as shown in Table I. Mouse lethality was observed in *A. carteri, A. klebsi, G. toxicus, O. siamensis, P. concavum,* and *P. lima.* The toxicity of *G. toxicus* to the mouse was outstanding. Hemolytic activity was most prominent in *A. carteri* and *A. klebsi,* although observed in all species tested. Potent ichthyotoxicity was observed in *A. carteri, A. klebsi,* and *P. concavum.*

The chromatographic and spectral analyses of PL toxin-2, the major toxin produced by *P. lima,* proved it to be okadaic acid (Murakami *et al.*, 1982), a cytotoxic polyether fatty acid derivative previously isolated from sponges (Tachibana *et al.*, 1981). PL toxin-1 was found to be a mixture of diol esters of okadaic acid. The structures of okadaic acid and the major ester in the PL toxin-1 fraction are shown in Figure 1. PL toxin-3 was shown to be a tertiary amine having a molecular weight of 981 (*m*/*z*). Elucidation of its chemical structure is under way.

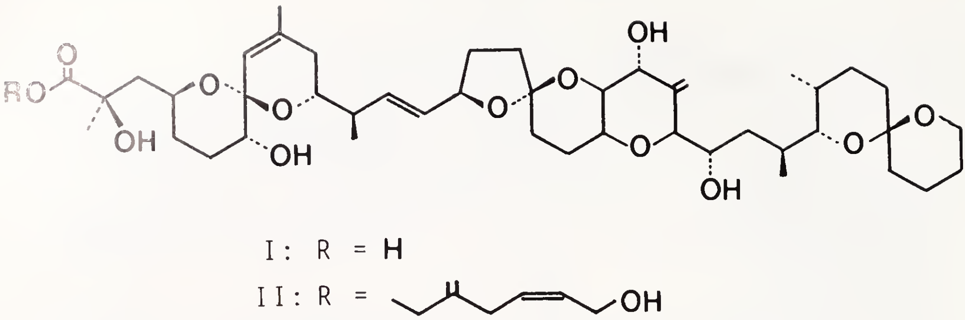

FIGURE 1. Okadaic acid (I) and one if its diolesters (II) from *Prorocentrum lima.*

Among the five hemolysins of *A. carteri,* hemolysin-1 and -2 were more abundant than the other three. The structures of hemolysin-1 and -2 were determined to be O-β-D-galactopyranosyl-(1-1)-3-O-octadecatetraenoyl-D-glycerol and O-α-D-galactosyl-(1-6)-O-β-D-galactopyranosyl-(1-1)-3-O-octadecatetraenoyl-D-glycerol, respectively (Fig. 2). The hemolytic activities of hemolysin-1 and -2 were 80% and 25% of that of the commercial saponin (Merck), respectively. The hemolytic activities of hemolysin-3, -4, and -5 were 100, 9, and 2 times more potent than the commercial saponin, respectively. Their chemical structures were indicated to be entirely different from those of hemolysin-1 and -2. Further structural work is under way.

Maitotoxin judged to be homogeneous by HPLC and TLC was obtained as an amorphous solid. It was extremely lethal to mice (0.13 μg/kg, ip). Mass spectra suggested the molecular weight of 3402 ± 2 (*m/z*). Chemical and spectral analyses indicated the absence of any amino acid or fatty acid moieties in the molecule. Further analyses of the structure are underway.

## DISCUSSION

The occurrence of diverse toxins in benthic dinoflagellates was confirmed. Out of the nine species tested, six produced mouse-lethal toxins, three ichthyotoxins, and all species produced hemolytic substances. Such a high occurrence of toxins is a charac-

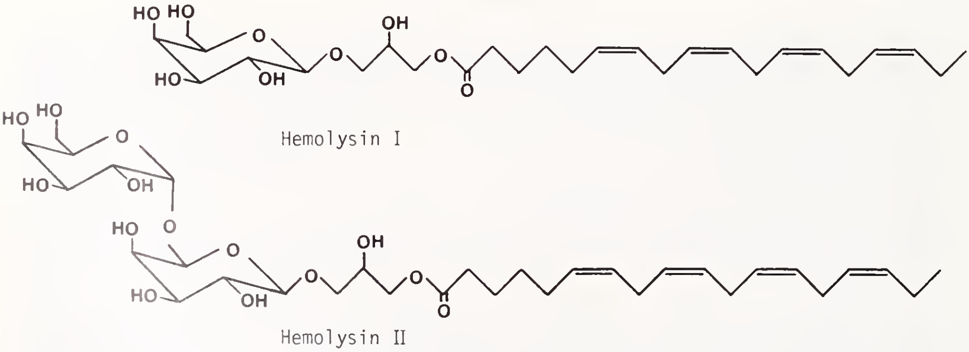

FIGURE 2. Hemolysin I and II from *Amphidinium carteri.*

teristic feature of benthic dinoflagellates. The biological and ecological significance of the toxins are not clear at present. Whether the toxins deter the growth of other microorganisms and thus benefit the elaborators remains to be tested.

The monoacylgalactolipids (hemolysin-1 and -2) are closely related to the known intermediate metabolites of photosynthesis, and therefore are likely to be widely distributed. The compounds may not be involved in ciguatera, but they could be responsible, in part, for fish kills during blooms of dinoflagellate species with no known ichthyotoxins.

Production of okadaic acid by *P. lima* is interesting because of its chemical resemblance to ciguatoxin and its potent diarrheagenicity. As *P. lima* is widely and densely distributed in coral reefs, there is a possibility that the compound, like ciguatoxin, is taken up by herbivorous fish and thus contributes to the diarrhea which is frequently seen in ciguatera patients.

The presence of maitotoxin in the viscera of surgeonfish has already been confirmed, and the toxin has been suspected of contributing to the diverse ciguatera symptoms seen in patients who have eaten herbivorous fish without first eliminating the viscera. The most characteristic feature of maitotoxin is its high lethality to mice (0.13 $\mu$g/kg, ip), which is 70 times that of saxitoxin or tetrodotoxin. The specific action of maitotoxin, to enhance the calcium ion influx through excitable membranes, was first discovered by Ohizumi's group (Takahashi *et al.,* 1982; Ohizumi *et al.,* 1983). Today the compound is being used extensively as a chemical tool in biomedical research.

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# PHARMACOLOGICAL ACTIONS OF THE MARINE TOXINS CIGUATOXIN AND MAITOTOXIN ISOLATED FROM POISONOUS FISH

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## ABSTRACT

Ciguatoxin and maitotoxin isolated from poisonous fish and toxic dinoflagellates exhibited a powerful excitatory effect on smooth and cardiac muscle. The ciguatoxin-induced excitatory action is due to an increase in $Na^+$ permeability of tetrodotoxin-sensitive Na channels, while the maitotoxin-induced excitatory effect is caused by an increased $Ca^{2+}$ permeability of the cell membrane.

## INTRODUCTION

A wide range of pharmacologically useful substances has been isolated from marine organisms. Among them, marine toxins such as tetrodotoxin (TTX), saxitoxin and sea anemone toxins, have been extensively studied since they affect a specific physiological function (Catterall, 1980). The first of these toxins, TTX, was isolated from puffer fish. It specifically blocks voltage-sensitive Na channels (Narahashi, 1974). Thus, TTX has been an essential tool in elucidating neurally mediated physiological processes. Such neurotoxins as TTX and saxitoxin, which bind with high affinity to Na channels, have proved to be useful probes for purifying and identifying these channels (Catterall, 1984).

Ciguatera is a disease caused primarily by the ingestion of any of a variety of fish inhabiting tropical and subtropical seas. The clinical symptoms for ciguatera are prickling of the lips, tongue, and throat; numbness; nausea; vomiting; metallic taste; dryness of the mouth; abdominal cramps; and diarrhea. It is well known that the principal toxin in ciguatera fish is ciguatoxin (CTX), a hydrohobic compound (Hashimoto, 1979). CTX is a highly toxic substance; the 50% lethal dose in mice is 0.45 $\mu$g/kg when injected intraperitoneally (i.p.) (Tachibana, 1980). Recently, maitotoxin (MTX), a water-soluble substance, has been isolated from the viscera of a surgeonfish, *Ctenochaetus striatus,* and from the toxic dinoflagellate *Gambierdiscus toxicus;* it was found to be the most potent marine toxin known. The minimum lethal dose of MTX in mice is 0.17 $\mu$g/kg (i.p.) which is approximately fifty times more potent than that of TTX (Yasumoto, 1980). The chemical structure of MTX has been only partially determined although it is considered to be a non-peptidic compound with a high molecular weight (Yasumoto, 1980).

## MATERIALS AND METHODS

### *Mechanical response*

Male guinea-pigs (250–350 g) and male rats (280–300 g) were used. The guinea-pig vas deferens and left atria were excised and mounted vertically in a 20 ml organ bath containing an Krebs-Ringer bicarbonate solution. The procedures for preparing the vas deferens (Ohizumi and Shibata, 1980) and the left atria (Kobayashi *et al.,* 1985a, b) were carried out as previously described. Cardiac myocytes were isolated from the rat using the method of Miyakoda and Nakamura (1982) and driven by a

TABLE I

*Effect of CTX on the NE release from the guinea-pig vas deferens in the presence or absence of TTX or $Ca^{++}$*

| Treatment | Amount of NE released[a] ng/g tissue/30 min |
|---|---|
| None | 39.3 ± 6.3 |
| CTX ($10^{-6}$ g/ml) | 1225.0 ± 240.8 |
| TTX ($5 \times 10^{-7}$ M)[b] + CTX ($10^{-6}$ g/ml) | 45.0 ± 9.7 |
| $Ca^{++}$-free medium[b] + CTX ($10^{-6}$ g/ml) | 82.8 ± 11.9** |

[a] Mean ± S.E. (n = 4).

[b] CTX was applied 15 min after treatment with TTX or incubation in $Ca^{++}$-free medium.

** Significantly different from the CTX-treated group, $P < .01$.

stimulator through a pair of platinum electrodes. Images of the cells were recorded either with a video recording system or with a high-speed movie camera.

### *Assay of norepinephrine (NE)*

Preparation and incubation of the guinea-pig vas deferens were performed as described previously (Norton *et al.,* 1981). The determination of released endogenous NE was carried out as described by Ohizumi *et al.* (1983).

### *Tissue Na and Ca content*

The Na (Ohizumi *et al.,* 1982) and Ca (Ohizumi *et al.,* 1983) concentrations in tissue were determined by the methods described earlier.

### *Na,K-ATPase assay*

Na,K-ATPase was purified from the porcine brain. The enzyme reaction was carried out at 37°C, for 20 min as described previously (Ohizumi and Yasumoto, 1983), and inorganic phosphate was determined according to the method of Martin and Doty (1949).

### *Extraction and purification of CTX and MTX*

CTX (Tachibana, 1980) and MTX (Yasumoto, 1980) were extracted and purified as described previously.

## RESULTS AND DISCUSSION

### *Smooth muscle*

CTX induced a dose-dependent contraction of the guinea-pig vas deferens at concentrations above $3 \times 10^{-7}$ g/ml (Ohizumi *et al.,* 1981). The CTX-induced contraction of the vas deferens was inhibited by phentolamine ($10^{-6}$ *M*), guanethidine ($10^{-4}$ *M*), reserpine (2 mg/kg/day, twice), TTX ($5 \times 10^{-7}$ *M*), or cold storage for 7 days at 4°C, but was not affected by atropine ($10^{-6}$ *M*) or mecamylamine ($3 \times 10^{-5}$ *M*). CTX ($1–6 \times 10^{-7}$ g/ml) caused a dose-dependent release of NE from the vas deferens, and the maximum response (1297.4 ng/g tissue) was obtained with a concentration of $6 \times 10^{-6}$ g/ml. The CTX-induced release of NE was nearly abolished by TTX (Table I) or by cold storage. These data suggest that the CTX-induced contraction of the vas deferens results from an indirect action mediated through NE release from the adrenergic nerve terminals, and that CTX causes an increase in $Na^+$ permeability of the presynaptic membrane, which may play an important role in its releasing action.

The dose-contractile response curves for the action of NE and KCl on the vas deferens were shifted to the left in a parallel manner by CTX, indicating that CTX caused supersensitivity (Ohizumi *et al.*, 1982). The CTX-induced potentiation was abolished by TTX ($5 \times 10^{-7}$ *M*) or saxitoxin ($5 \times 10^{-7}$ *M*); it was inhibited by a $Na^+$-deficient medium, but was not affected by phentolamine ($10^{-6}$ *M*). After treatment with reserpine (2 mg/kg/day, twice) or cold storage (4°C, for 7 days), CTX-induced release of NE was completely prevented. CTX still markedly potentiated the response to NE and KCl in the reserpinized preparation or the cold stored preparation. On the other hand, tissue Na content of the vas deferens was markedly increased by ouabain ($10^{-5}$ *M*), but was not affected by CTX at concentrations between 1 and $5 \times 10^{-7}$ g/ml. However, in the presence of ouabain ($10^{-5}$ *M*), Na content was increased 30% or more by CTX ($5 \times 10^{-7}$ g/ml). This increasing effect of CTX on Na content was abolished by TTX ($10^{-6}$ *M*) (Ohizumi *et al.*, 1982). These data suggest that CTX causes an increase in $Na^+$ permeability across the TTX-sensitive Na channels of smooth muscle cells, and this may play an important role in its mechanism of potentiation.

The responses of the guinea-pig vas deferens to MTX were different from those of CTX. MTX ($10^{-9}$ to $3 \times 10^{-8}$ g/ml) caused a dose-dependent slower contraction of the vas deferens (second phase) that followed the initial rapid phasic contraction (first phase) (Ohizumi *et al.*, 1983). The second component of the MTX-induced contraction was markedly inhibited by phentolamine ($10^{-6}$ *M*) and reserpine (2 mg/kg/day, twice), whereas the first component remained unaffected. Both components were inhibited or abolished by verapamil ($10^{-6}$ to $10^{-5}$ *M*) or $Ca^{2+}$-free medium, but were not affected by atropine ($10^{-6}$ *M*), chlorpheniramine ($10^{-6}$ *M*), or TTX ($10^{-6}$ *M*). The tissue Ca content of the vas deferens was increased by MTX ($10^{-9}$ to $3 \times 10^{-8}$ g/ml) in a dose-dependent manner. Furthermore, MTX ($10^{-9}$ to $3 \times 10^{-8}$ g/ml) caused a dose-dependent release of NE from the vas deferens, which was inhibited or abolished by verapamil ($3 \times 10^{-6}$ and $10^{-5}$ *M*) or $Ca^{2+}$-free medium, but not by TTX ($10^{-6}$ *M*). In $Na^+$-free medium, MTX still caused a marked increase in NE release from the tissue (Table II). It has been reported that MTX elicited Ca-dependent excitatory effects on neuronal cells (Takahashi *et al.*, 1982, 1983; Freedman *et al.*, 1984). These observations suggest that the major part of the first component of the MTX-induced contraction is the result of a direct action of MTX on the smooth muscle membrane, whereas the second component is primarily the result of indirect action mediated through the NE release from the adrenergic nerve terminals. The data also suggest that $Ca^{2+}$, but not $Na^+$ is indispensable for the action of MTX.

In the isolated guinea-pig and rat ventricle strips, MTX ($10^{-10}$ to $4 \times 10^{-9}$ g/ml) caused a dose-dependent inotropic effect. The MTX-induced inotropic effect was nearly abolished by $Co^{2+}$ (2 m*M*) or verapamil ($3 \times 10^{-7}$ *M*), but was little affected by propranolol ($10^{-6}$ *M*), reserpine (2 mg/kg, twice), or TTX ($5 \times 10^{-7}$ *M*) (Kobayashi *et al.*, 1985). The tissue Ca-content of guinea-pig left atria was increased by MTX (2–3 $\times 10^{-8}$ g/ml), and this increase was markedly inhibited by $Co^{2+}$ (2 m*M*) or verapamil ($10^{-5}$ *M*). In rat myocardial cells, MTX ($10^{-10}$ to $10^{-9}$ g/ml) induced an increase in the degree and the rate of contraction and subsequent arrhythmogenic actions (Kobayashi *et al.*, 1985). These effects of MTX were antagonized by verapamil ($10^{-6}$ *M*) and were completely inhibited by $Ca^{2+}$-free medium. When the concentration of external $Ca^{2+}$ was increased to 2 m*M*, MTX ($10^{-8}$ g/ml) made all the rod cells turn into arrhythmically moving cells and then into rod-shaped cells much faster than was the case in the control medium. These results suggest that the excitatory effect of MTX on heart muscle is caused by a direct action on the cardiac muscle membrane, due mainly to an increase in $Ca^{2+}$ permeability, possibly through some Ca channels.

TABLE II

*Effect of MTX on the NE release from the guinea-pig vas deferens in the presence or absence of TTX or verapamil*

| Solution<br>Treatment | Amount of NE released[a] ng/g tissue/30 min |
|---|---|
| Normal solution | |
| None | 22 ± 4 |
| MTX (3 × $10^{-8}$ g/ml) | 1350 ± 169 |
| MTX (3 × $10^{-8}$ g/ml) + TTX (5 × $10^{-7}$ M)[b] | 1397 ± 205 |
| MTX (3 × $10^{-8}$ g/ml) | |
| + verapamil (3 × $10^{-6}$ M)[b] | 770 ± 81* |
| + verapamil ($10^{-5}$ M)[b] | 548 ± 62* |
| $Ca^{++}$-free solution[c] | |
| None | 18 ± 3 |
| MTX (3 × $10^{-8}$ g/ml) | 97 ± 11 |
| $Na^{+}$-free solution[c] | |
| None | 832 ± 54 |
| MTX (3 × $10^{-8}$ g/ml) | 2973 ± 134 |
| MTX (3 × $10^{-8}$ g/ml) + verapamil ($10^{-5}$ M)[b] | 2010 ± 110* |

[a] Mean ± S.E. (n = 6).

[b] TTX and verapamil were added 15 min before the application of MTX.

[c] Tissue were incubated for 15 min in $Ca^{++}$- or $Na^{+}$-free solution before the application of TTX or verapamil.

* Significantly different from MTX alone in each solution, $P < .01$.

Finally, scaritoxin, a hydrohobic compound isolated from a ciguatera fish *Scarus gibbus,* has a releasing action of NE and ACh from adrenergic and cholinergic nerve endings, resulting in the contraction of the guinea-pig vas deferens and ileum, respectively (Tatsumi *et al.,* 1985).

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# THE USE OF THE MOSQUITO BIOASSAY FOR DETERMINING THE TOXICITY TO MAN OF CIGUATERIC FISH

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## ABSTRACT

Moderately well-correlated with the mouse bioassay, requiring a much smaller amount of raw fish flesh, and employing a faster chemical extraction procedure, the mosquito bioassay represents significant progress in detecting ciguatoxin in fish muscle. We use the mosquito bioassay to evaluate the approximate amount of ciguatoxin present in the remains of 25 fish of 17 different species involved in 99 documented cases of ciguatera poisoning. From the data provided by mosquito bioassay and human symptomatology, we determine a dose-effect relationship for ciguatoxin (CTX) to man. Three average pathogenic doses (PD) are established. The minimum PD is estimated to be 0.6 ng CTX/kg, the PD50 to be 2 ng CTX/kg, and the PD100 to be 8 ng CTX/kg. These data suggest a MLD (minimal lethal dose) value of 20 ng CTX/kg.

## INTRODUCTION

Ciguatera is the most frequently reported food-borne disease in French Polynesia. Its sporadic and scattered outbreaks, tied to natural and human phenomena, constitute a widespread and serious problem in these islands, both as a health hazard (Fig. 1) and as an obstacle to the exploitation of reef-fish resources (Fig. 2).

As accurate an evaluation as possible of ciguatera levels is imperative for use by local authorities. Until a simple, reliable, rapid chemical assay, like the one recently proposed by Hokama (1985), is available, bioassays will continue to be widely used. Usually, small animals are used for bioassays. Thus, the mosquito bioassay (Pompon *et al.,* 1984), which has a moderate correlation to the cat bioassay and the mouse bioassay (Bagnis *et al.,* 1985), represents significant progress in detecting ciguatoxin in fish muscle. The swiftness of the chemical extraction procedure (Pompon and Bagnis, 1984) and of the mosquito response, as well as the small amount of fish needed, allow us to check the ciguatoxicity of very small fish (Chungue *et al.,* 1984) or of the remains of poisonous meals. The following study aims to establish, from the data taken from the mosquito bioassay and human symptoms, a relationship between the approximate amount of ingested ciguatoxin (Scheuer *et al.,* 1967) and its clinical effects (Bagnis *et al.,* 1979).

## MATERIAL AND METHODS

### *Patients*

Patients either were seen in the consulting room of the Louis Malarde Medical Research Institute in Papeete or were hospitalized in the General Hospital in Papeete. Clinical symptoms were observed in all the poisoned people.

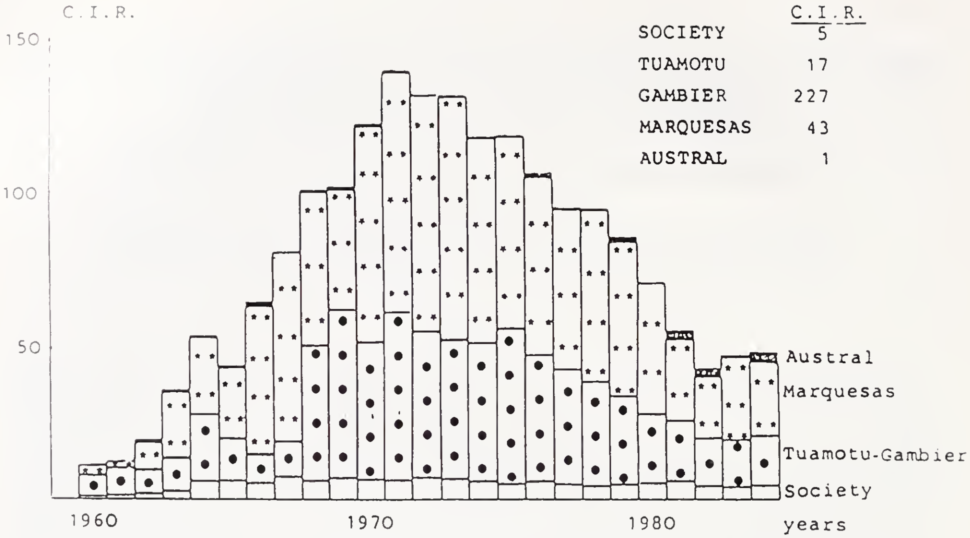

FIGURE 1. Geographical distribution of the incidence of ciguatera per 1000 inhabitants (C.I.R.) in French Polynesia (1960 to 1984).

### *Fish*

Fish belonged to 17 species at several trophic levels and were caught in various areas of French Polynesia. Each fish sample was responsible for at least one documented case of ciguatera. The average amount of fish flesh eaten by an individual was evaluated by anamnesis and by weighing control rations.

### *Rapid extraction of the ciguatoxin*

Ciguatoxin extraction procedures are detailed in Figures 1 and 2. A sample (8 g) of ground raw or cooked fish flesh was homogenized with 40 ml of acetone (twice) and centrifuged. The supernatants were concentrated and percolated through a cartridge of diatomaceous silica (4 g) that was washed successively by n-hexane (45 ml) and diethyl ether (45 ml). After evaporation, the diethyl ether toxic residue was subjected to fast, low-pressure chromatography on silicic acid (1 g). Elution was performed successively by chloroform (25 ml) and a chloroform-methanol mixture (9:1:25 ml). The resulting eluate was evaporated. From 8 g of fish flesh, 1.0 ± 0.2 mg of toxic residue was obtained within 1 h, regardless of the species or the toxicity of the fish.

### *Mosquito bioassay*

The mosquitoes, weighing 1.6 ± 0.2 mg, belonged to the species *Aedes aegypti.* They were intrathoracically injected as described by Rosen and Gubler (1974). Each portion of extract obtained from 8 g of flesh was suspended in 100 $\mu$l of phosphate-buffered saline (pH 7.4, 0.002 $M$) containing 0.5% gelatin and 5% heated calf serum (56°C for 30 min). A volume of 0.5 $\mu$l of two-fold serial dilutions was injected into eight groups of ten mosquitoes each. The tested doses ranged from 0.31 to $40 \times 10^{-3}$ g of flesh per mosquito. A control group received diluent. Mortality was observed 1

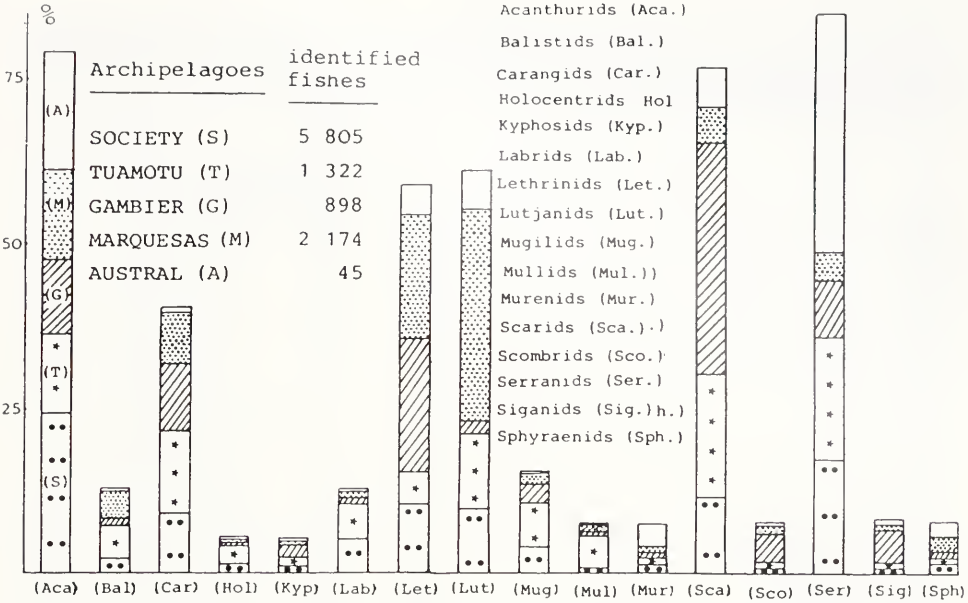

FIGURE 2. Geographical distribution of ciguateric fish in French Polynesia.

h after injection. The method of Bliss (1938) was used to calculate LD50 in mosquitoes. The fish ciguatoxicity was expressed in equivalent gram of fish (fge) or in *Aedes* mosquito-units (AU). A fish contains 1 AU of ciguatoxin when five mosquitoes in a group of ten die after each has received extract equivalent to 7 mg of fish.

### *Dose-effect relationship for ciguatoxin*

Using as a baseline the MLD of pure ciguatoxin for mice [*i.e.*, 0.45 g/kg according to Tachibana (1980)], the correspondence between cat bioassay (Bagnis and Fevai, 1971), mouse bioassay (Yasumoto *et al.*, 1977), and mosquito bioassay have been previously established (Bagnis *et al.*, 1985). They are as follows:

$$1 \text{ AU} = 17.5 \times 10^{-5} \text{ mouse-units (MU)} = 8.8 \times 10^{-5} \text{ cat units (CU)}$$
$$= 1.6 \times 10^{-3} \text{ ng CTX}.$$

## RESULTS AND DISCUSSION

### *Epidemiological features*

Twenty-five fish, eaten by 142 individuals, induced ciguatera symptoms in 99 people (see Table I). Among these, 30 were hospitalized for a period ranging from 24 hours to 10 days. The fish involved in the most severe cases were carnivorous fish from four species: *Lethrinus miniatus, Cheilinus undulatus, Aprion virescens,* and *Sphyraena barracuda.*

### *Clinical features*

The most frequently observed symptoms are shown in Table II. Sensory disturbances were much more frequent than neuromotor symptoms.

TABLE I

*Results of the mosquito bioassay for 25 fish involved in documented cases of ciguatera poisoning, with the corresponding toxicological data provided from the mouse bioassay and the cat bioassay*

| | Man | | Mouse | Cat | Mosquito | | |
|---|---|---|---|---|---|---|---|
| Fish | (a) | (b) | (c) | (d) | (e) | (f) | (g) |
| *Lethrinus miniatus* (Tautira) | 6/8 | 3 | 0.23 | 0.12 | 1.02 | 6.9 | 1.54 |
| *Lethrinus miniatus* (Raiatea) | 8/10 | | 1.00 | 0.18 | 2.70 | 2.6 | 0.58 |
| *Lethrinus miniatus* (Vairao) | 3/5 | | 0.35 | 0.10 | 3.11 | 2.3 | 0.50 |
| *Lethrinus miniatus* (Paea) | 9/9 | 3 | 0.90 | 0.14 | 1.64 | 4.3 | 0.96 |
| *Cheilinus undulatus* (Arue) | 6/6 | 6 | 1.20 | 0.28 | 0.89 | 7.9 | 1.77 |
| *Cheilinus undulatus* (Paea) | 6/6 | 2 | 2.25 | 0.23 | 1.44 | 4.9 | 1.10 |
| *Cephalopholis argus* (Tautira) | 7/7 | 5 | 0.50 | 0.10 | 2.30 | 3.0 | 0.68 |
| *Cephalopholis argus* (Teahupoo) | 2/2 | | 0.60 | 0.15 | 1.61 | 4.4 | 0.98 |
| *Epinephelus microdon* (Mataiva) | 4/7 | | 0.18 | 0.11 | 2.05 | 3.4 | 0.77 |
| *Sphyraena barracuda* (Moorea) | 7/7 | | 0.60 | 0.12 | 2.02 | 3.5 | 0.78 |
| *Aprion virescens* (Marquesas) | 6/6 | 6 | | 0.14 | 0.83 | 8.4 | 1.90 |
| *Naso unicornis* (Mataiva) | 2/2 | | 0.52 | 0.10 | 2.65 | 2.6 | 0.57 |
| *Scarus jonesi* (Hao) | 5/5 | | 2.25 | 0.14 | 0.98 | 7.1 | 1.60 |
| *Scarus jonesi* (Mataiva) | 3/3 | | 1.80 | 0.18 | 1.53 | 4.6 | 1.03 |
| *Lutjanus bohar* (Cook) | 3/3 | 3 | 0.29 | 0.18 | 2.37 | 3.0 | 0.67 |
| *Lutjanus bohar* (Tetiaroa) | 6/8 | | 0.45 | 0.10 | 2.23 | 3.1 | 0.71 |
| *Monotaxis grandoculis* (Mataiva) | 2/2 | | 0.36 | | 1.45 | 4.8 | 1.09 |
| *Sphyraena barracuda* (Rairoa) | 5/5 | 2 | 0.74 | 0.10 | 2.22 | 3.3 | 0.74 |
| *Lutjanus gibbus* (Papeete) | 2/5 | | 0.84 | | 6.30 | 1.1 | 0.26 |
| *Caranx melampygus* (Punaauia) | 1/6 | | 0.18 | 0.01 | 3.14 | 2.2 | 0.50 |
| *Plectropomus leopardus* (Taenga) | 1/8 | | 0.09 | 0.10 | 4.77 | 1.5 | 0.33 |
| *Gymnosarda unicolor* (Gambier) | 2/5 | | 0.30 | 0.02 | 6.19 | 1.1 | 0.26 |
| *Lutjanus gibbus* (Papeete) | 1/6 | | 0.09 | | 6.66 | 1.1 | 0.24 |
| *Crenimugil crenilabis* (Mataiva) | 1/5 | | 0.15 | | 7.95 | 0.9 | 0.19 |
| *Scarus gibbus* (Hao) | 1/5 | | 0.10 | | 7.11 | 1.0 | 0.22 |

*LEGEND:* (a) Number of patients/number of consumers.
(b) Number of hospitalized patients.
(c) Dose of ciguatoxin in nanograms per gram of fish (CTX:ng/fg) estimated by the mouse bioassay.
(d) CTX (ng/fg) estimated by the cat bioassay.
(e) LD50 in $10^{-3}$ fge per 1.6 mg mosquito.
(f) LD50 in mosquito-units (AU).
(g) CTX (ng:fg) estimated by the mosquito bioassay.

Of the 30 hospitalized patients, 24 displayed chiefly cardiovascular symptoms. Bradycardia with a pulse ranging from 40 to 60 beats/min (21% of cases), systolic blood pressure of 100 mm Hg or less (18% of cases), abnormalities of electrocardiograms (15% of cases) and cardiac distress (6% of cases) were observed. Twelve patients were hospitalized for other severe symptoms which may or may not have been associated with cardiovascular disturbances: ataxia (10), paresis (2), dyspnea (8), and dysuria (5).

TABLE II

*Frequency of symptoms in 99 documented cases of ciguatera*

| Symptom | % of patients with finding* |
|---|---|
| 1. Paresthesia of the extremities | 92 |
| 2. Circumoral pareshesia | 92 |
| 3. Burning or pain to skin on contact with cold water | 91 |
| 4. Arthralgia | 88 |
| 5. Myalgia | 86 |
| 6. Diarrhea | 74 |
| 7. Asthenia | 66 |
| 8. Nausea | 56 |
| 9. Pruritus | 48 |
| 10. Headache | 45 |
| 11. Abdominal pain | 44 |
| 12. Chills | 43 |
| 13. Vomiting | 40 |
| 14. Bradycardia | 38 |
| 15. Vertigo | 33 |
| 16. Perspiration | 32 |
| 17. Ataxia | 28 |
| 18. Hypotension | 28 |
| 19. Dental pain | 24 |
| 20. Tremor | 18 |
| 21. Salivation | 18 |
| 22. Watery eyes | 17 |
| 23. Dyspnea | 16 |
| 24. Cardiac dysrhythmia | 15 |
| 25. Neck stiffness | 13 |
| 26. Dysuria | 13 |
| 27. Paresis | 8 |
| 28. Cardiac distress | 6 |
| 29. Hiccoughs | 2 |

* Percentages in table refer to all 99 documented ciguatera cases. Percentages in text refer to symptoms observed in the 30 hospitalized cases.

### *Toxicological features*

The data on fish ciguatoxicity, as determined by the mosquito bioassay, are shown in Table I. Also indicated are the corresponding doses of ng CTX, evaluated from the cat bioassay and the mouse bioassay results when available. We could not correlate a high level of ciguatoxicity in a fish to a severe syndrome in all its consumers.

For a more accurate analysis, the toxic fish were separated into two classes. Eighteen fish, poisonous for at least 50% of their consumers, were labelled ≥PD50 fish. Their ciguatoxicity ranged from $0.83 \times 10^{-3}$ fge (inferior toxicity, IT) to $6.3 \times 10^{-3}$ fge (superior toxicity, ST) with a mean toxicity (MT) evaluated to be $1.83 \times 10^{-3}$ fge, from 1.1 to 8.4 AU (MT = 4.2), and from 0.26 to 1.9 ng CTX (MT = 1). Seven fish, poisonous for less than 50% of the consumers, were labelled <PD50 fish. For them, the ciguatoxicity ranged from 3.14 to $7.95 \times 10^{-3}$ fge (MT = $6.02 \times 10^{-3}$), from 0.9 to 2.2 AU (MT = 1.3), and from 0.19 to 0.5 ng CTX (MT = 0.9).

### *Dose-effect relationship for ciguatoxin in man*

In the 25 documented cases mentioned above, the average ration of fish eaten by the patients was estimated to be 0.30 ± 0.15% of their body weight (62 ± 18 kg);

TABLE III

*Average and extreme doses of ciguatoxin per kg of individual weighing 62 kg and consuming 0.3% of the body weight (186 g) of poisonous fish from mouse, cat, and mosquito bioassays, expressed in animal-units and in nanograms*

| Dose of ingested CTX | Mouse bioassay | | Cat bioassay | | Mosquito bioassay | | Average dose of CTX (ng) from the three bioassays |
|---|---|---|---|---|---|---|---|
| | MU | ng | CU | ng | AU | ng | |
| | | | (Batch of 18 ≥ LD50 fish) | | | | |
| Maximal | 2 | 18 | $4.5 \times 10^{-2}$ | 0.8 | $3.6 \times 10^{-3}$ | 5.7 | 8.2 |
| Mean | $4 \times 10^{-1}$ | 3.2 | $2 \times 10^{-2}$ | 0.4 | $1.6 \times 10^{-3}$ | 2.6 | 2.1 |
| Minimal | $6 \times 10^{-2}$ | 0.5 | $1.5 \times 10^{-2}$ | 0.3 | $0.9 \times 10^{-3}$ | 0.5 | 0.8 |
| | | | (Batch of 7 < LD50 fish) | | | | |
| Maximal | $3 \times 10^{-1}$ | 2.5 | $3 \times 10^{-2}$ | 0.3 | $1.0 \times 10^{-3}$ | 1.5 | 1.4 |
| Mean | $8 \times 10^{-2}$ | 0.8 | $2 \times 10^{-2}$ | 0.1 | $0.6 \times 10^{-3}$ | 0.9 | 0.6 |
| Minimal | $3 \times 10^{-2}$ | 0.3 | $0.3 \times 10^{-2}$ | 0.03 | $0.5 \times 10^{-3}$ | 0.6 | 0.3 |

*i.e.*, about 186 g. The doses indicated in Table III were calculated on this basis. The maximum, minimum, and mean doses of CTX toxic to man were obtained from the maximum (ST), minimum (IT), and average (MT) ciguatoxicity per gram of fish as previously defined.

The results of the mosquito bioassay were compared to those of the cat bioassay and the mouse bioassay.

(1) The PD50, expressed in ng CTX per kg of man, ranged from 0.5 to 18 (MD = 3.2) in the mouse bioassay, from 0.3 to 0.8 (MD = 0.4) in the cat bioassay, and from 1.5 to 5.7 (MD = 2.6) in the mosquito bioassay. The mean of the three bioassay values, which is considered to be the definitive PD50, ranged from 0.8 to 8.2 (MD = 2.1).

(2) The doses of CTX per kg of body weight which made at least one, but less than 50%, of the consumers sick were tentatively labelled minimum pathogenic doses (MPD). They ranged from 0.3 to 2.5 (MD = 0.8) in the mouse bioassay, from 0.3 to 3.0 (MD = 0.1) in the cat bioassay and from 0.6 to 1.5 (MD = 0.9) in the mosquito bioassay. The definitive MPD ranged from 0.3 to 0.9 (MD = 0.5).

Everyone who ate the two most toxic fish, which caused the most severe cardiovascular symptoms, required hospitalization. Considering the approximate amount of fish ingested in those cases, the PD100 for man can be estimated at 8 ng CTX/kg.

## CONCLUSIONS

Ciguatoxin is one of the most potent biotoxins—via the intraperitoneal route—to mice. It also appears to be one of the most toxic natural substances to man when consumed orally. It was possible to determine a dose-effect relationship for ciguatoxin to man from the data provided when the mosquito bioassay was applied to the leftovers of 25 fish involved in 99 cases of ciguatera poisoning. Three average pathogenic doses (PD) have been defined. The minimum PD is estimated to be 0.6 ng CTX/kg (*i.e.*, 0.7 MU/kg); the PD50 is estimated to be 2 ng CTX/kg (*i.e.*, 0.3 MU/kg); and the PD100 is estimated to be 8 ng/kg (*i.e.*, 0.9 MU/kg). These data suggest a MLD for man of approximately 20 ng CTX/kg (*i.e.*, 3 MU/kg).

## ACKNOWLEDGMENTS

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# ASSESSMENT OF A RAPID ENZYME IMMUNOASSAY STICK TEST FOR THE DETECTION OF CIGUATOXIN AND RELATED POLYETHER TOXINS IN FISH TISSUES

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## ABSTRACT

We have developed a rapid and simple enzyme immunoassay stick test for ciguatoxin and related polyether toxins in fish tissues. This assay can be used to examine clinically implicated fishes from ciguatera poisonings, corresponding catches of fishes obtained in the same vicinity as those clinically implicated, non-toxic consumed fishes, and nearshore reef fishes. With this assay, 14 clinically documented fishes gave a mean and standard deviation ($\bar{x}$ ± S.D.) of 3.1 ± 0.7. The corresponding catches of fishes from areas implicated in ciguatera poisonings showed the following percentages of toxicity for *Ctenochaetus strigosus:* Hawaii Island, 45.0%; Kauai, 80.6%; and Oahu, 42.5%. Sixty non-toxic consumed fishes gave a $\bar{x}$ ± S.D. value of 1.2 ± 0.5, and *Thunnus thynnus,* a pelagic fish never implicated in ciguatera poisoning, gave a $\bar{x}$ ± S.D. value of 1.1 ± 0.8. All of the clinically implicated fishes were in the borderline to positive value ranges with the stick test. Associated with the high incidence of toxicity of *C. strigosus* from Kauai, *Gambierdiscus toxicus* was found in the gut of all 93 fish samples examined. The stick enzyme immunoassay has proven to be simple, rapid, sensitive, and specific.

## INTRODUCTION

Ciguatera poisoning, endemic to the tropics and subtropics and a health problem to the consumer, is associated with the consumption of ciguatoxin, a low dalton lipid polyether (Scheuer *et al.,* 1967; Tachibana, 1980; Nukina *et al.,* 1984). The origin of this toxin is the dinoflagellate *Gambierdiscus toxicus* (Yasumoto *et al.,* 1977; Adachi and Fukuyo, 1979). Dinoflagellates also produce other structurally related polyether toxins such as okadaic acid (Tachibana *et al.,* 1981; Murakami *et al.,* 1982) and brevetoxin (Lin *et al.,* 1981; Baden *et al.,* 1981).

Okadaic acid was isolated from the dinoflagellate *Prorocentrum lima* (Murakami *et al.,* 1982) as well as from the sponges in the genus *Halichondria* (Tachibana *et al.,* 1981), and brevetoxin was extracted from the dinoflagellate causing red tide, *Ptychodiscus brevis* (*Gymnodinium breve*) (Lin *et al.,* 1981; Baden *et al.,* 1981). These polyether toxins appear to have an effect on the membranes of nerve and muscle tissues and to induce changes in ion permeability of the cells (Rayner, 1972; Miyahara *et al.,* 1979; Ohizumi *et al.,* 1982).

A major area of study initiated in the past decade has been the development of sensitive and specific assay methods for the assessment of these toxins in contaminated fish tissues. Earlier assays included: (1) the feeding of fish tissues to cats and mongoose (Banner *et al.,* 1961; Bagnis, 1973); and (2) the injection of crude extracts into mice, chicks, and, more recently, mosquitoes (Kosaki *et al.,* 1968; Yasumoto *et al.,* 1971; Kimura *et al.,* 1982; Chungue *et al.,* 1984). These tests lacked both sensitivity and specificity. More recent studies have taken the immunological approach, using antibodies prepared in sheep and rabbit, following immunization with conjugates of the polyether toxins (Hokama *et al.*, 1977; Kimura *et al.*, 1982; Hokama *et al.*, 1983, 1984, 1985; Baden *et al.*, 1984).

We have developed and assessed a rapid, simple, specific, and sensitive stick test to detect ciguatoxin and related polyether toxins in contaminated fish tissues. The fishes examined included: (1) clinically implicated fishes from ciguatera poisonings; (2) fishes from corresponding catches obtained in the same vicinity with the implicated fishes; (3) portions of non-toxic fishes that were consumed without incident; and (4) fishes from the nearshore waters of Hawaii where toxicity occasionally occurs.

## MATERIALS AND METHODS

### *Sources of fish samples*

The following fishes were obtained through the courtesy of the Department of Health (DOH), State of Hawaii: Clinically implicated fish samples from ciguatera poisonings—1, *Acanthocybium solandri;* 1, *Aprion virescens;* 3, *Caranx* species; 2, *Cephalopholis argus;* 1, *Sphyraena* species; and 1, grouper extract. Corresponding catch fishes associated with the fishes implicated in ciguatera poisonings—93, *Ctenochaetus strigosus;* 16, *Mulloidichthys* species; 6, *Cephalopholis argus;* 3, *Cheilinus rhodochrous;* and 1, *Aphareus furcatus.*

The following fishes were obtained through the courtesy of the Department of Land and Natural Resources (DLNR), State of Hawaii: Non-toxic consumed fishes—43, *Seriola dumerili;* 6, *Elagatis bipinnulatis;* 5, *Sphyraena* species; and 1, *Caranx* species. Reef fishes from the nearshore waters of Oahu and Hawaii Island—*Cheilinus rhodochrous; Kyphosus cinerescens; Ctenochaetus strigosus; Lutjanus kasmira; Acanthurus* species; and *Myripristis* species.

Non-toxic consumed *Thunnus thynnus* was obtained from commercial sources (supermarket).

### *Source of purified ciguatoxin*

Purified ciguatoxin isolated from livers of moray eels (Nukina *et al.*, 1984) was obtained through the courtesy of Professor P. J. Scheuer of the Department of Chemistry, University of Hawaii, Honolulu, Hawaii.

### *Stick test reagents*

The reagents for the stick test were similar to those used in the enzyme immunoassay reported previously (Hokama *et al.*, 1983). These included: (a) methanol fixative, 0.3% hydrogen peroxide ($H_2O_2$) in absolute methanol, prepared fresh daily; (b) Tris buffer A, 0.05 $M$ Tris (hydroxymethyl)aminomethane, pH 7.5 ± 0.05, with 0.1% human serum albumin (HSA) and 0.01% sodium azide; (c) Tris buffer B, prepared as in A, but without HSA and sodium azide; (d) sheep-anti-CTX coupled to horseradish peroxidase (HRP, Type VI, RZ:3:3, Sigma Chemical Co., St. Louis, MO) according to the one-step glutaraldehyde method (Voller *et al.*, 1980), stored in aliquots of 1 ml at −20°C. until ready for use; (e) substrate, 25 ml of 0.3% $H_2O_2$ in Tris buffer B added to 10 mg of 4-chloro-1-naphthol previously dissolved in 0.125 ml of absolute methanol, mixed thoroughly then filtered through Whatman #1 filter paper, prepared fresh just before use; (f) bamboo sticks (length, 200.0 mm; diameter, 2.5 mm; Mum's Taisei of Hawaii, Honolulu) with the skewer ends coated with Liquid Paper (The

Gillette Co., Rockville, MD). Any excess Liquid Paper was removed in the preparation of the coat, then the coating was allowed to dry thoroughly before use.

### *Stick test procedure*

Fish were sampled by inserting the skewer end of the stick coated with the Liquid Paper into the dorsal-anterior and ventral-posterior sections of one or both sides of the fish. Each stick was inserted 5 times into the flesh at 1 s/insertion. Each fish was examined with six sticks; three in the dorsal-anterior and three in the ventral-posterior portions of the side of the fish. The stick was air dried and then immersed into the fixative for 1 s without shaking. The excess solution was blotted onto tissue paper and the stick was air dried. The stick was then washed in Tris buffer B thoroughly with gentle shaking for 10 s and the excess solution blotted onto tissue paper. The stick was then immersed for 15 to 30 s without agitation into the sheep-anti-CTX-HRP solution previously diluted 1:200 with Tris buffer A just prior to use. The excess antibody conjugate was blotted onto tissue paper. The stick was then washed in two changes of Tris buffer B, 10 s each with gentle shaking. After the excess buffer was blotted, the stick was immersed into 0.3 ml of the substrate. The tube containing the substrate and the stick was shaken, then incubated for 10 min at room temperature. The intensity of the color was compared with a color scheme as follows: 0, essentially no color; 1.0+, slightly bluish-purple; 1.5 to 2.0+, lightly bluish-purple; 2.0 to 2.5+, moderately bluish-purple; 3.0 to 5.0+, moderately to intensely bluish-purple. The results of these reactions were scored as follows: 0 to 2.0+, negative; 2.1 to 2.4+, borderline; and values greater than 2.5+, positive. The fractional values were the result of averaging several sticks per sample.

The reagents for the test were arranged in the following sequence: (a) fixative; (b) Tris buffer B wash 1; (c) sheep-anti-CTX-HRP; (d) Tris buffer B wash 2; (e) Tris buffer B wash 3; and (f) substrate.

### *Analysis of purified ciguatoxin by the stick test*

Purified ciguatoxin in concentrations of 0, 1, 5, and 25 ng/ml in absolute methanol were prepared. The coated sticks were immersed for 2 s in the ciguatoxin-methanol solutions. The sticks were then air dried and examined by the stick test procedure. The fixation step was not performed for the purified toxin. Several sticks were used for each concentration of the toxin solution. The mean and standard deviation of the stick values were obtained for each toxin concentration and plotted as shown in Figure 1.

### *Examination of the gut contents of* Ctenochaetus strigosus

Smears on microslides from the viscera of *C. strigosus* were prepared from each of the 93 specimens obtained from Kauai as part of the corresponding catches. The smears were suspended in 50% glycerol phosphate buffer saline with a cover slip. Each smear was examined at 400× magnification with the phase and light microscope. Twenty fields per smear were examined and the average number of *Gambierdiscus toxicus* per field determined.

All statistical evaluation was determined by the method of Tallarida and Murray (1981).

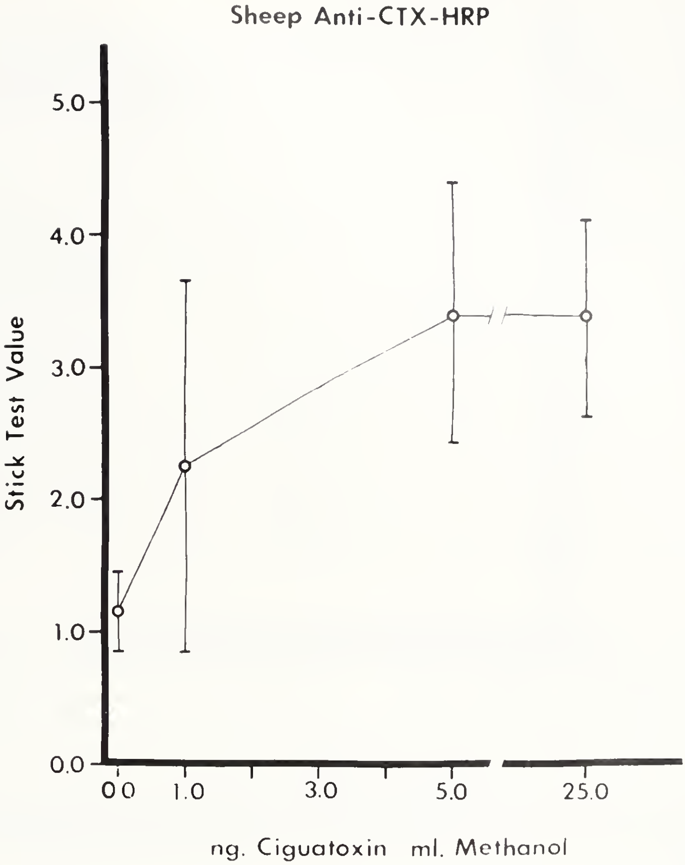

FIGURE 1. Analysis of purified ciguatoxin in methanol by the stick test. The y-axis represents the stick test values and the x-axis the concentration of ciguatoxin. The means (O) and standard deviations (vertical lines) are presented.

## RESULTS

### *Stick test analysis of positive fishes from clinically documented ciguatera poisoning and negative consumed fishes*

The assessment of the validity of the stick test in the detection of toxic fishes is summarized in Figure 2. Sixteen clinically implicated raw and cooked (tissues, soup, and gravy) fish samples examined by the stick test gave a $\bar{x} \pm$ S.D. of 3.3 ± 0.7 while 60 non-toxic consumed fishes gave a $\bar{x} \pm$ S.D. of 1.2 ± 0.5 stick test value (Fig. 2). The difference between these two categories was highly significant with $P < 0.005$. Examination of 2 samples of *Thunnus thynnus* gave a stick test value of 1.1 ± 0.8. This value was significantly different from the positive values with $P < 0.01$. *Thunnus thynnus,* a pelagic fish, has not been implicated in ciguatera poisoning. On the basis of this study, the interpretation of the stick test values has been derived and designated as follows: 0–2.0+, negative; 2.1–2.4+, borderline; and values greater than 2.5+ as positive (Fig. 2).

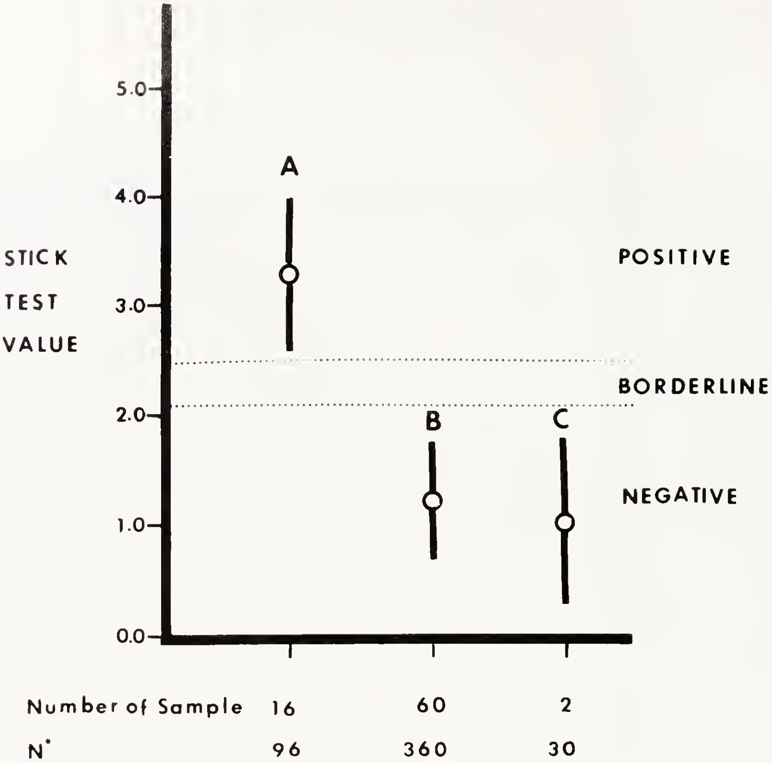

FIGURE 2. Comparison of clinically documented positive fishes associated with ciguatera poisoning (A) and non-toxic consumed negative fishes (B) by the stick test. (C) Represents the *Thunnus thynnus* results. The y-axis represents the stick test values and the x-axis the number of samples. n* = the number of determinations.

### *Stick test analysis of corresponding catches of fishes and study of the nearshore reef fishes of Hawaii*

The results of examining, by the stick test, several species of fishes obtained as corresponding catches with fishes implicated in ciguatera poisonings are shown in Table I. Of the 120 fishes from 4 species examined, 69.2% were considered toxic by the stick test. The positive group also included those fishes in the borderline category. This high percentage is due to *Ctenochaetus strigosus,* which caused a large outbreak of ciguatera poisoning on Kauai in August, 1984 (see Table II). The gut contents of all 93 samples contained 0.1 to 1.5 *Gambierdiscus toxicus* per field at 400× magnification (Fig. 3).

### *Summary of stick test evaluation of nearshore reef fishes from Oahu, Hawaii Island, and Kauai*

These studies are summarized in Tables II, III, and IV. All four species of fishes examined from Oahu came from the Leeward side of the nearshore waters (Table III). Fishes caught in these areas generally have the highest frequency of ciguatera poisoning, in contrast to the Windward side, where essentially no ciguatera poisoning

TABLE I

*Examination of several species of fishes from corresponding catches of ciguatera poisoning in Hawaii, 1984*

| Species | Total number | Stick test<br>Negative | <br>Positive |
|---|---|---|---|
| | | No. of fish | |
| *Ctenochaetus strigosus* (Kole, surgeon fish) | 93 | 18 | 75 |
| *Cephalopholis argus* (Roi, grouper) | 6 | 4 | 2 |
| *Mulloidichthys* species (Weke, goat fish) | 16 | 15 | 1 |
| *Cheilinus rhodochrous* (Po'ou, wrasse) | 3 | 0 | 3 |
| *Aphareus furcatus* (Wahanui, snapper) | 1 | 0 | 1 |
| Total | 120 | 37 | 81 |
| % | – | 30.8 | 69.2 |

has occurred. A high percentage of toxicity is shown by *C. strigosus* (43.5%) and *L. kasmira* (36.5%). Note that the herbivore (*C. strigosus*) gave a higher percentage of toxic samples than either the carnivore (*L. kasmira*) or the two other species examined (*Acanthurus* and *Myripristis*). The positive category includes the borderline samples.

Table IV summarizes the results of the samples of three species from Hawaii Island. A high percentage of positives is found in *C. strigosus* (45.0%) as compared to *K. cinerescens* (0.0%) and *C. rhodochrous* (20.8%).

TABLE II

*Comparison of* Ctenochaetus strigosus *examined by the stick test from three different islands: Hawaii, Oahu, and Kauai*

| Source | Total number | Stick test<br>Negative | <br>Positive |
|---|---|---|---|
| | | No. of fish | |
| Kauai* | 93 | 18 | 75 |
| | % | 19.4 | 80.6 |
| Hawaii** | 51 | 28 | 23 |
| | % | 55.0 | 45.0 |
| Oahu*** | 108 | 61 | 47 |
| | % | 56.5 | 43.5 |

* Samples from corresponding catches of ciguatera poisoning outbreak, August, 1984.

** Samples obtained in areas associated with ciguatera outbreaks from *Cephalopholis argus* and *Cheilinus rhodochrous*, April–August, 1984.

*** Samples from Barbers Point and Ewa, routine monthly survey of Harbor development and control site, 1984.

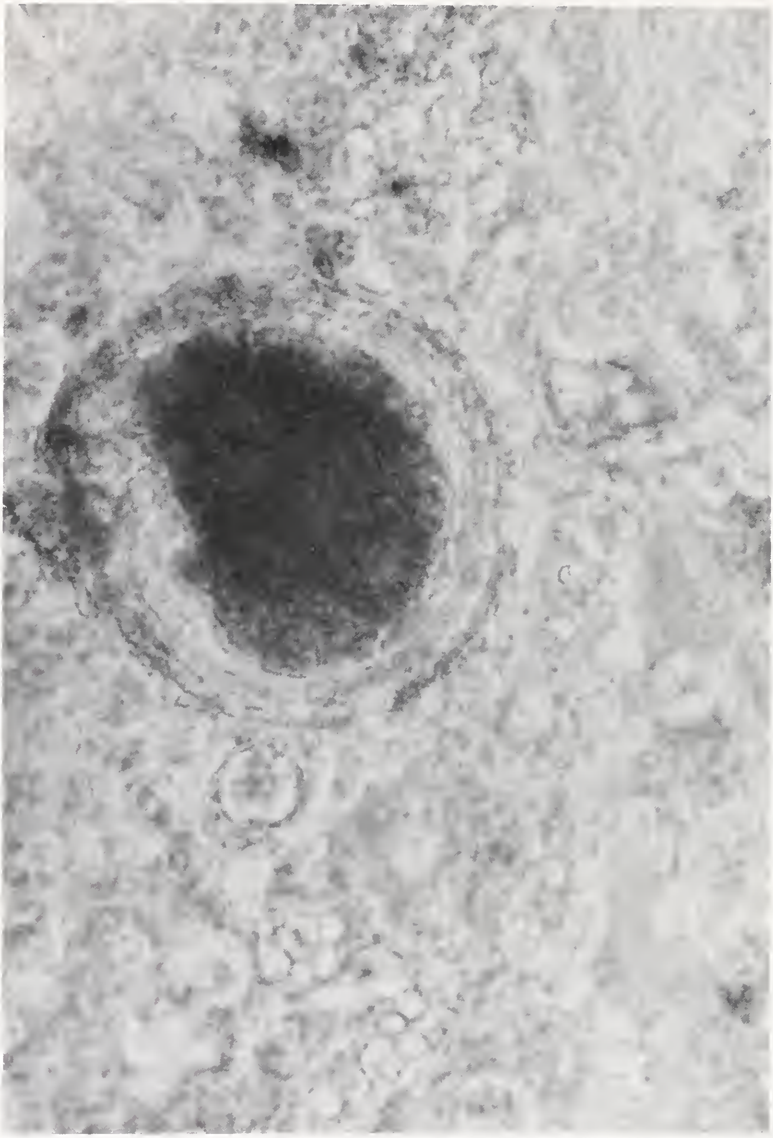

FIGURE 3. A typical photograph of *Gambierdiscus toxicus* from the gut of *Ctenochaetus strigosus* obtained from the nearshore waters of Kauai (400× magnification).

The comparison of *C. strigosus* from the three different islands by the stick test procedure is shown in Table IV. The highest percentage of toxicity was shown in samples from Kauai (80.6%). Samples from Oahu and Hawaii Island were nearly identical, 43.5% and 45.0%, respectively. This is understandable, since the samples from Kauai were part of the corresponding catches of a large ciguatera outbreak due to *C. strigosus*. The Hawaii Island samples of *C. strigosus* were caught on the Leeward side in April of 1984 just prior to small outbreaks of ciguatera poisoning that contin-

TABLE III

*Summary of stick test assessment of fishes from Oahu*

| Species | Total number | Stick test<br>Negative | <br>Positive |
|---|---|---|---|
| | | No. of fish | |
| *Ctenochaetus strigosus* | 108 | 61 | 47 |
| (Kole, surgeon fish) | % | 56.5 | 43.5 |
| *Lutjanus kasmira* | 96 | 61 | 35 |
| (Taape, blue-line snapper) | % | 63.5 | 36.5 |
| *Acanthurus* species | 16 | 19 | 1 |
| (Palani, surgeon fish) | % | 76.0 | 24.0 |
| *Myripristis* species | 12 | 9 | 3 |
| (Menpachi, squirrel fish) | % | 75.0 | 25.0 |
| Total | 241 | 149 | 91 |
| % | – | 61.8 | 38.2 |

ued throughout 1984. The fishes implicated included *Cephalopholis argus, Ctenochaetus strigosus,* and *Cheilinus rhodochrous.* The Oahu samples of *C. strigosus* were obtained during the autumn of 1984 from the DLNR Barbers Point (Leeward side of Oahu) study. No incidence of ciguatera poisoning was reported during this period (an outbreak occurred in January, 1985, in this area). In part, this may be attributable to the lack of fishing in this area due to the turbid water conditions caused by the deep harbor dredging and construction at Barbers Point.

### *Microscopic examination of viscera smears from* C. strigosus *(Kauai)*

All 93 samples of *C. strigosus* showed *Gambierdiscus toxicus* in their gut contents when smears were examined with the phase microscope at 400× magnification. The range of dinoflagellates/field was 0.1 to 1.5. A typical example of *G. toxicus* is shown in Figure 3.

TABLE IV

*Summary of stick test assessment of fishes from Hawaii*

| Species | Total number | Stick test<br>Negative | <br>Positive |
|---|---|---|---|
| | | No. of fish | |
| *Ctenochaetus strigosus* | 51 | 28 | 23 |
| (Kole, surgeon fish) | % | 55.0 | 45.0 |
| *Kyphosus cinerescens* | 28 | 28 | 0 |
| (Nenue, rudder fish) | % | 100.0 | 0.0 |
| *Cheilinus rhodochrous* | 24 | 19 | 5 |
| (Po'ou, wrasse) | % | 79.2 | 20.8 |
| Total | 103 | 75 | 28 |
| % | – | 72.8 | 27.2 |

## DISCUSSION

The results of this study show the feasibility of adapting the enzyme immunoassay to a simple and rapid stick test. This procedure retained its sensitivity and specificity using the conventional heterologous sheep-anti-CTX-HRP (Hokama *et al.,* 1977) or the recently prepared monoclonal antibodies to toxic polyethers (Hokama *et al.,* 1985). The success of the procedure is probably due to the selective adsorption or attraction of the lipid toxins in fish tissues by one or more constituents in the Liquid Paper coated onto the bamboo stick. The latter alone without coating had no activity in the assay. Coated sticks alone have shown essentially little activity with values equal to or below the normal value (less than 2.0+). This is attributable in part to the non-specific binding of the antibody enzyme conjugate.

We compared the clinically implicated toxic fishes and the non-toxic consumed fishes with the stick test and demonstrated significant differences in the mean and standard deviation of the stick test values between these two groups with $P < 0.005$. These same samples tested by the enzyme immunoassay (Hokama *et al.,* 1983, 1984, 1985) agreed with the stick test values presented in this report. Hokama *et al.,* (1984) recently showed that the sheep-anti-CTX-HRP detected ciguatoxin and structurally related polyether toxins such as okadaic acid and brevetoxin in a competitive enzyme immunoassay procedure. The stick test also interacted with purified ciguatoxin in a dose-responsive manner (Fig. 1).

Examination of the fishes from corresponding catches showed a high level of toxicity (80.6% positive), especially *Ctenochaetus strigosus* from the island of Kauai during August, 1984, when an outbreak of ciguatera poisoning occurred involving 14 individuals. At least one and up to four *C. strigosus* were consumed by each individual. The viscera of all 93 specimens retrieved from the markets by the DOH contained *Gambierdiscus toxicus.* Outbreaks of ciguatera poisoning occurred in Hawaii during most of 1984, beginning in the early spring months. A moderate to high level of toxicity was also noted in *C. strigosus* (45.0%); with a slightly lower level in *Cheilinus rhodochrous* (21.0%). However, *Kyphosus cinerescens* essentially showed no toxicity though caught in the same area. The toxicity patterns of Oahu appeared similar to that of the island of Hawaii (Tables II and IV).

The radioimmunoassay (Hokama *et al.,* 1977), the enzyme immunoassay (Hokama *et al.,* 1983, 1984), and the stick test (Hokama, 1985) all use the immunological approach for the detection of ciguatoxin and related polyether toxins directly from fish tissues. The stick test differs from the former two tests in its simplicity and rapidity. The stick test also requires no capital equipment, and it can be used at home or in the field. Such a system also may be used for other enzyme immunoassays, perhaps using other coats to selectively adsorb the antigenic or haptenic components onto the stick. Furthermore, the stick test does not require the extraction of tissues, as required for the mouse bioassay (Yasumoto *et al.,* 1971; Kimura *et al.,* 1982) and the recently described mosquito assay (Chungue *et al.,* 1984). However, the stick test is also capable of examining fish tissue extracts. In addition, the stick test can be performed rapidly, while retaining its sensitivity and specificity, for mass screening of fishes on a large commercial level with minimal costs.

In summation, the stick test is simple, rapid, and inexpensive, with sufficient specificity and sensitivity to evaluate the levels of ciguatoxin and related polyether toxins in fishes suspected of potential ciguatera poisoning.

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*The Biological Bulletin* accepts outstanding original research reports of general interest to biologists throughout the world. Papers are usually of intermediate length (10–40 manuscript pages). Very short papers (less than 10 manuscript pages including tables, figures, and bibliography) will be published in a separate section entitled "Short Reports." A limited number of solicited review papers may be accepted after formal review. A paper will usually appear within four months after its acceptance.

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# THE EFFECTS OF EMBRYOS OF DIFFERENT DEVELOPMENTAL STAGES ON REPRODUCTIVE BEHAVIOR AND PHYSIOLOGY IN BROODING FEMALES OF THE AMPHIPOD CRUSTACEAN *GAMMARUS PALUSTRIS*

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## ABSTRACT

The expression of male reproductive behavior is correlated with female intermolt stage in the amphipod crustacean *Gammarus palustris.* Previous studies suggested that three factors that vary with female intermolt stage influence males: (1) waterborne pheromones, (2) contact pheromones on females' exoskeleta; and (3) female behavior. The present study was conducted to determine whether a fourth factor, developmental stage of brooded embryos, might also affect male behavior. In addition, the effects of the embryos on the females' own behaviors and physiologies were examined.

The contents of the females' brood pouches were altered by removing the broods of females of specific intermolt stages, and presenting the females with embryos of other females. *G. palustris* females readily place conspecifics' embryos in their pouches. Thus males' responses to females at the same intermolt stage but with broods of different developmental stages could be observed.

The data showed that the nature of the females' brood pouches had no measurable effect on male behavior. In addition, the embryos' ages did not affect female intermolt period, reproductive behavior, or reproductive physiology.

## INTRODUCTION

In some species with brood care, the presence of a brood can influence adult reproductive behavior and physiology. This has been best documented in the vertebrates and the social insects. For example, the presence of embryos in the nest of the stickleback, *Gasterosteus aculeatus,* inhibits male courtship behavior (Sevenster-Bol, 1962), and the removal of embryos from a nest of the great tit, *Parus major,* can induce courtship behavior and the production of another brood (Lack, 1966). Further, the presence or absence of eggs clearly influences egg production in at least several social insects (Wilson, 1971).

The crustaceans typically exhibit brood care, but the relationship between the embryos and reproductive behavior and physiology has not been examined in most marine species. An exception to this is the observation that the removal of the embryos from the brood pouches of female *Gammarus lawrencianus* (an amphipod) stimulates some aspects of the males' reproductive behavior (Dunham, 1986). This is an interesting discovery because the typical *Gammarus* life-history pattern (described below) suggests both the feasibility and the advantage of this potential method of regulating reproductive effort.

*Gammarus* females typically produce several broods in succession during the

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warmer months in the temperate zone. Generally, amplexus (precopulation) begins towards the end of the female's intermolt period and continues until the female molts, at which time copulation occurs. Then, within an hour, ovulation occurs, and the male and female separate. All the embryos of the current brood are deposited in the female's brood pouch simultaneously at ovulation. Development occurs in the pouch, and the juveniles hatch and emerge shortly before the female is due to molt again [the times of hatching and emergence are species-specific (Borowsky, 1980b)]. The female remains alone until a few days before her next molt, when amplexus is reinitiated and the cycle is repeated.

Thus, all the embryos of a specific brood are at the same stage of development, the stage of development of the embryos is correlated with the female's intermolt period, and males generally initiate amplexus when the brood is in the later stages of development. Further, males should have little difficulty in determining the nature of the brood pouch contents because the pouch is open to the environment (Borowsky, 1980a), and the embryos could stimulate male behavior either by direct contact, or by water-borne substances.

Earlier studies had shown that *Gammarus palustris* females will readily place the broods of other conspecifics in their own pouches (Borowsky, 1983). This suggested that the effects of brood pouch contents on male behavior could be examined in detail in this species by exchanging embryos between females of different intermolt stages.

It was reasoned that the presence of broods could influence reproductive behavior of both sexes in several ways. First, immature embryos might inhibit these behaviors; second, mature embryos might stimulate them; and third, both of these possibilities might be true. In addition, if the embryos had some effect, it could either be direct, in which case the mere presence of the embryos would influence behavior, or it could be indirect, first modifying the female's physiology over the course of days, and then altering both sexes' behaviors. In the first case, the presence or absence of embryos would alter the animals' behaviors within a short time, but in the second case, some time would be necessary for the physiology of the female to change.

## MATERIALS AND METHODS

All animals employed in the present study were obtained by hand-picking individuals from under rocks and debris in the intertidal zone at low tide at Jamaica Bay, New York, on 2 and 4 April 1986. Animals were brought to the laboratory immediately, and couples in amplexus were placed in individual 10 cm diameter glass culture dishes in water from the collecting site (about 25 ppt). They were maintained at 20°C with a light cycle of 15:9 L:D, and supplied with *Ulva lactuca* thalli *ad libitum*.

Females in amplexus and carrying embryos in the later stages of development in their brood pouches were classed as "receptive." Previous studies had shown that the intermolt period of *G. palustris* females is about eight days at 20°C, and that females who had molted two days before rarely entered into amplexus. Therefore, females who had molted and ovulated two days before and were not in amplexus were classified as "non-receptive" in this study. Eight experiments were conducted as follows: non-receptive females were assigned at random to four treatment groups; Experiment I, females were placed on frozen seawater; Experiment II, females whose embryos were removed were presented with the embryos of other non-receptive females; Experiment III, females whose embryos were removed were not presented with any embryos; Experiment IV, females were presented with receptive females' embryos. Receptive females were randomly assigned to the other four groups: Experiment V, females were placed on frozen seawater; Experiment VI, females whose embryos were removed were presented with the embryos of other receptive females; Experiment VII, females whose embryos were removed were not presented with any embryos; and Experiment VIII, females whose embryos were removed were presented with the embryos of non-receptive females (Tables I, II). Each experiment employed ten different pairs of animals (80 tests were conducted altogether).

Embryos were removed as follows: females were placed on a bed of frozen seawater to anesthetize them, then the embryos were aspirated from the pouch with a fine pipette inserted between the brood plates. After the embryos were removed, females were placed in a clean dish with fresh seawater and allowed to recover for one day. Depending on the experiment, the females were either presented with their own embryos, with other females' embryos, or with no embryos at all (as described above). Females presented with embryos placed them in their brood pouches during the one-day recovery period.

The responses of males to females were tested after the recovery period (24 h after treatment) as follows: ten females from each experimental treatment were tested individually by placing the female and a male simultaneously in a clean culture dish with fresh seawater. Each male was obtained by gently separating it from another female immediately before a test. Three observations were made: first, the time from the introduction of the animals to the dish and the occurrence of "grabbing" behavior (the male grasps the female's exoskeleton firmly with its claws); second, the time from grabbing to either the initiation of amplexus or the separation of the couple ("contact time"); and third, the occurrence of amplexus.

"Grabbing" was noted because this is the earliest behavior in the typical sequence of reproductive behaviors which indicates unequivocally that the male is aware of the presence of the female, and "contact time" was measured because it represents the time during which the male obtains the information from the female which determines whether he will initiate amplexus (Borowsky and Borowsky, 1985, 1987). Amplexus is stereotyped and is easily distinguished from other behaviors (Borowsky, 1984).

After the behavioral observations, the couples were maintained in individual dishes until the female either molted or died. This was done for two reasons; first, to verify the viability of removed and replaced embryos by making sure they developed and hatched; and second, to determine whether the presence of embryos whose developmental stage was inappropriate for the female's intermolt period influenced behavior later in the female's cycle. Accordingly, after each couple's behavior was observed, the animals were observed daily, noting the day that the brood hatched, the day when amplexus was initiated, and the day of the female's molt.

## RESULTS

There was a significant difference among the eight experiments in the number of times that amplexus was initiated ($\chi^2_7 = 52.4$, $P < 0.001$). The difference was due to female class ("receptive" *versus* "non-receptive"), rather than to the nature of the brood pouch contents. There was a significant difference between all non-receptive females grouped together and all receptive females grouped together regardless of brood pouch contents (Tables I and II, respectively; one of 40 non-receptive females entered into amplexus, while 32 of 40 receptive females entered into amplexus; $\chi^2_1 = 49.6$, $P < 0.001$). In contrast, there was no significant difference between females with broods and those without (33 of 60 females with embryos engaged in amplexus, and 10 of 20 females without embryos engaged in amplexus; $\chi^2_1 = 0.15$, $P > 0.05$). It is especially noteworthy that there was no significant difference among the four exper-

TABLE I

*Male reproductive behaviors expressed to non-receptive females whose brood pouches had different contents*

| Experiments | Time to grab (s) $\bar{x} \pm SD$ | Time to grab (s) Range | Time to decision (s) $\bar{x} \pm SD$ | Time to decision (s) Range | Decisions Number of amplexes (of 20 tests) |
|---|---|---|---|---|---|
| I. Ice-treated: embryos not removed | 122.7 ± 127 | 4–446 | 11.5 ± 12 | 1–39 | 0 |
| II. Embryos exchanged between non receptive females | 155.7 ± 142 | 31–516 | 14.7 ± 19 | 0–59 | 0 |
| III. Embryos removed permanently | 176.0 ± 175 | 2–442 | 33.3 ± 56 | 0–164 | 0 |
| IV. Embryos removed and replaced with receptive females' embryos | 86.3 ± 92 | 0–285 | 33.1 ± 41 | 2–147 | 1 |

iments performed on each class of female (non-receptive and receptive females, Tables I and II, respectively: $\chi^2_3$s = 3.08 and 3.50, respectively; *P*s > 0.05). Thus, the nature of the contents of the females' brood pouches did not affect the frequency of amplexus.

In addition, the lengths of time from the introduction of pairs of animals into observation dishes to the times when males grabbed females was not significantly different among the eight experiments (Tables I and II: one-way ANOVA; $F^7_{68} = 1.55$, $P > 0.05$). Further, there was no significant difference among the groups in the lengths of time from grabbing to either amplexus or separation (one-way ANOVA; $F^7_{72} = 1.76$, $P > 0.05$). Thus, while the decision to engage in amplexus depended largely upon whether the female was receptive, the length of time employed to arrive at the decision was not.

Another effect of the brood on reproductive behavior could be to modify the female's physiology. The frequency of amplexes increases as female intermolt period advances, but there is variability among individual females' initial day of amplexus (Borowsky and Borowsky, 1985, 1987). It was hypothesized that the absence of a brood might accelerate the time to amplexus, thus enhancing the successful fertilization of the next brood. However, there was no significant difference in the time from the female's molt to the onset of amplexus between non-receptive females whose broods had been removed permanently and non-receptive females who retained their

TABLE II

*Male reproductive behavior expressed to receptive females whose brood pouches had different contents*

| Experiments | Time to grab (s) $\bar{x} \pm SD$ | Time to grab (s) Range | Time to decision (s) $\bar{x} \pm SD$ | Time to decision (s) Range | Decisions Number of amplexes (of 20 tests) |
|---|---|---|---|---|---|
| V. Ice-treated: embryos not removed | 26.5 ± 22 | 4–79 | 59.2 ± 44 | 16–180 | 7 |
| VI. Embryos exchanged between receptive females | 148.1 ± 164 | 9–553 | 41.0 ± 38 | 14–147 | 8 |
| VII. Embryos removed permanently | 55.7 ± 44 | 3–126 | 41.1 ± 16 | 15–76 | 10 |
| VIII. Embryos removed and replaced with non-receptive females' embryos | 103.9 ± 112 | 4–329 | 31.5 ± 23 | 2–74 | 7 |

broods (Experiment III *vs.* Experiments I and II combined, respectively; ns = 10 and 20, ranges 8–18 and 9–22, and $\bar{x}$ = 12.8 ± 4 and 13.6 ± 4, respectively: Student's *t*-test; $t_{28} = -0.4625$).

Another way the absence of a brood might influence female physiology could be to accelerate the time until the female's next molt. However, the intermolt periods of females without broods were about the same as the intermolt periods of females with broods (Experiment III *vs.* Experiments I and II combined, respectively: ns = 10 and 17, ranges 12–23 and 12–25 days, and $\bar{x}$s = 16.9 ± 4 and 16.9 ± 4, respectively).

## Discussion

The data show that on the basis of the criteria employed in the present study, the nature of the contents of females' brood pouches has no effect on reproductive behavior or reproductive physiology in *G. palustris.* In receptive females, the absence of broods did not increase, and the presence of immature broods did not decrease the frequency of amplexus. In the reciprocal experiments on non-receptive females, the presence of a mature brood did not increase the frequency of amplexus. Finally, the absence of a brood did not significantly hasten the onset of amplexus in non-receptive females without broods nor did it accelerate the time to these females' next molt.

These data are consistent with earlier observations of *G. palustris* reproductive behaviors which suggest that the females' behaviors and contact pheromone(s) on their exoskeleta stimulate amplexus and copulation (Borowsky and Borowsky, 1985, 1987).

But the results differ from those of Dunham (1986) who found that *G. lawrencianus* females with empty brood pouches engaged in amplexus more often than females carrying broods, even when they were at a comparable intermolt stage. There are several explanations for the differences. First, two different species were studied. Significant differences in behavior have been found between other *Gammarus* species (Borowsky, 1980b). But second, it is possible that the slightly different criteria employed for scoring amplexus accounts for the difference in results. Occasionally a male *G. palustris* carried a female in the amplexus position immediately after an encounter but dropped it shortly thereafter. Since the function of amplexus is to maintain pairing until fertilization is possible, this temporary amplexus was not considered to be biologically significant and was not scored here. Dunham's observation consisted of noting whether the pair was in amplexus three minutes after the animals were introduced to each other.

It would be interesting to test the effects of broods on the reproductive behavior and physiology of the larger Crustacea. The larger species generally reproduce infrequently [for example the lobster, *Homarus americanus,* reproduces biennially, (Aiken and Waddy, 1980)], and would stand to benefit from a mechanism which compensated for the loss of a current brood.

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# SEXUAL REPRODUCTION AND COLONY GROWTH IN THE SCLERACTINIAN CORAL *PORITES ASTREOIDES*

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## ABSTRACT

This study examines patterns of sexual reproduction and colony growth for *Porites astreoides* Lamarck, an abundant Caribbean reef coral. Five factors influence the reproductive condition of this coral in Jamaica: (i) Season, (ii) lunar day, (iii) polyp location within a colony, (iv) colony size, and (v) colony age. *P. astreoides* has an unusual mixed breeding system: Approximately half of colonies are hermaphroditic and half are female. Although gonads occur in some colonies throughout the year, there are clear seasonal differences both in the number of reproductive colonies within the population and in colony fecundity. Male gametes are spawned monthly around the time of the full moon. The abundance and maturity of brooded larvae peaks prior to the new moon. Within reproductive colonies, gonads and brooded larvae are more abundant in central polyps than at colony edges. Among female colonies, the onset of reproduction is apparently related to colony size, whereas the fecundity of individual polyps is related to colony age. Hermaphroditic and female colonies differ in the size at which most colonies are reproductive. Rates of vertical and lateral growth for *P. astreoides* increase with colony size, but not with colony age. These findings demonstrate how the combined effects of several variables can cause individuals within a population to differ greatly in reproductive condition, fecundity, and growth rate.

## INTRODUCTION

Since the turn of the century, there have been numerous descriptions of the patterns of reproduction and growth among reef corals (*e.g.,* Duerden, 1902; Wood-Jones, 1910; Vaughan, 1915; Marshall and Stephenson, 1933; Atoda, 1947; Harrigan, 1972; Connell, 1973; Loya, 1976; Gladfelter *et al.,* 1978; Stimson, 1978; Rinkevich and Loya, 1979a, b; Hughes and Jackson, 1980, 1985; Goreau *et al.,* 1981; Kojis and Quinn, 1981, 1982; Harriott, 1983; Highsmith, 1982; van Moorsel, 1983; Babcock, 1984; Harrison *et al.,* 1984; Jokiel *et al.,* 1985; Shlesinger and Loya, 1985; Stoddart and Black, 1985; Szmant-Froelich *et al.,* 1985; Wallace, 1985; Kinzie and Sarmiento, 1986; Szmant, 1986). It is becoming clear from some of these studies that co-occurring colonies of a given species can differ greatly in important characteristics such as reproductive state, fecundity, and growth rate (Marshall and Stephenson, 1933; Connell, 1973; Stimson, 1978; Rinkevich and Loya, 1979a, b; Harriott, 1983;

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Kojis and Quinn, 1981, 1984, 1985; Hughes and Jackson, 1985; Stoddart and Black, 1985; Szmant-Froelich, 1985; Wallace, 1985).

Such variation can be caused by external events such as environmental stress. For example, coral fecundity may decline due to injury (Kojis and Quinn, 1981), exposure to sedimentation or turbidity (Kojis and Quinn, 1984), or competitive encounters (Rinkevich and Loya, 1985). Similarly, rates and patterns of coral growth may be affected by colony breakage (Loya, 1976).

Other intraspecific differences in reproduction and growth are more inherent to populations, resulting from relatively predictable factors such as ontogenetic stage, season, or lunar period. For example, in protandrous hermaphroditic species, small and/or young colonies are male while larger and/or older colonies are hermaphroditic (Rinkevich and Loya, 1979a; Kojis and Quinn, 1981). Among species in which the onset of reproduction is related to colony size, including certain gorgonian octocorals (Wahle, 1983) and scleractinian corals (Rinkevich and Loya, 1979b; Kojis and Quinn, 1981, 1985; Babcock, 1984; Szmant-Froelich, 1985), mature colonies will stop producing gametes when reduced in size below the reproductive threshold value, irrespective of their age (Wahle, 1983; Kojis and Quinn, 1985; Szmant-Froelich, 1985). Polyp fecundity has been shown to vary with colony size (Rinkevich and Loya, 1979b; Kojis and Quinn, 1981; Babcock, 1984), with colony age (Kojis and Quinn, 1985), with polyp size (Harriot, 1983), and with polyp location within a colony (Harrigan, 1972; Rinkevich and Loya, 1979b; Wallace, 1985). Finally, both the abundance and developmental stage of coral gonads or brooded larvae can change temporally with season or with lunar period (*e.g.*, Harriot, 1983; Kojis and Quinn, 1984; Stoddart and Black, 1985).

When individuals of a species vary greatly, estimates of reproductive condition, fecundity, or growth rate based on small sample sizes may not accurately reflect patterns of reproduction and growth within the population as a whole. Furthermore, interspecific comparisons may be problematic when intraspecific differences equal or exceed those occurring between species. Nevertheless, remarkably few studies have systematically evaluated the levels and possible sources of intraspecific variation in reproduction or growth (*e.g.*, Connell, 1973; Rinkevich and Loya, 1979b; Kojis and Quinn, 1981, 1984, 1985; Harriot, 1983; Babcock, 1984; Hughes and Jackson, 1985; Szmant-Froelich, 1985; Kinzie and Sarmiento, 1986).

This paper documents intraspecific patterns of sexual reproduction and growth within a population of the reef coral *Porites astreoides* Lamarck. Specifically, we describe: (i) Seasonal and lunar patterns in sexual reproduction; (ii) intra-colony variation in polyp fecundity and gender; (iii) the effects of colony age on polyp fecundity; (iv) the relationship between colony size and reproductive condition; and (v) changes in colony growth rate that occur with increasing colony size.

## MATERIALS AND METHODS

### *Study organism*

*Porites astreoides* is among the most common corals on many Caribbean reefs and occurs over a wide range of depths and habitats (Goreau, 1959; Goreau and Wells, 1967). It is generally considered to be among the most fecund species, since juveniles appear early and abundantly in studies of coral recruitment (Bak and Engel, 1979; Rylaarsdam, 1983; Rogers *et al.*, 1984). *P. astreoides* colonies grow in encrusting, platey, and mound-like morphologies. Corals sampled in this study had encrusting to plate-like shapes, as these predominate in the study population at −10 m on the west forereef of Discovery Bay, Jamaica. At this site, instances where injury has subdivided coral colonies into physiologically separate, but genetically identical "daughter" colonies (Hughes and Jackson, 1980, 1985) were usually clearly identifiable by the color similarity, close proximity, or remaining skeletal connections between adjacent colonies. Previous surveys suggest that most colonies (78%) in this population result from the independent recruitment of larvae rather than from the subdivision of larger colonies (Chornesky, unpub. data).

### *Sexual reproduction*

Sexual reproduction was evaluated between October 1981 and July 1982 by histologically examining coral tissues for gonads and larvae. Our sampling scheme was designed to reveal both seasonal and lunar patterns in reproductive periodicity. Tissues were sampled throughout one lunar period (28 days) during each of four months that reflect the annual range of seawater temperature: July (high); January (low); April (intermediate, increasing); and October (intermediate, decreasing). During each of these four months, samples were collected every five or six days (a total of five sampling days per month). On each sampling day, separate tissue specimens of thirty or more polyps were collected from both the centers and growing edges of five *P. astreoides* colonies. Colonies sampled on any given day were spatially separated by distances of several meters, and thus can be safely assumed to be of different genotypes. Individual colonies were sampled only once during the study, to avoid any adverse effects of repeated injury on coral reproduction (*e.g.,* Kojis and Quinn, 1981). In total, 100 colonies were sampled (4 months × 5 days × 5 colonies).

The general condition, size, and maximum skeletal thickness of each colony was recorded. Colony size was determined by calculating the surface area of live coral tissues (the area of an ellipse defined by the two largest, perpendicular diameters laid across the colony's living tissues). Maximum skeletal thickness was measured when possible (59% of colonies sampled).

For encrusting and plate-shaped corals, maximum thickness provides a better measure of colony age than surface area. Surface area is often only poorly correlated with colony age since injury along colony perimeters frequently interrupts or inhibits the lateral expansion of corals (Jackson, 1979; Hughes and Jackson, 1980, 1985). In contrast, colony centers are less likely to be injured and upward skeletal growth in this region may proceed relatively uninterrupted. The growth discrepency between colony regions is often reflected in colonies of equal maximum thickness but widely varying surface area (Chornesky, unpub. data). Thus, maximum skeletal thickness is the most reliable historical marker of previous growth, and is used here to estimate minimum colony age.

Results of tests for size differences of sampled corals among sampling days and among sampling months by one-way ANOVA were non-significant (among days, $F = .98$, $P > .25$, df = 19,80; among months, $F = .18$, $P > .75$, df = 3,96). Similarly, colony thickness did not differ significantly among sampling months ($F = .77$, $P > .5$, df = 3,58). The numbers of colonies for which thickness could be measured did not allow for statistical comparison among sampling days. Colony size and thickness were not significantly correlated for the population of corals sampled ($r = .22$, $P > .05$).

To minimize the potential effects of uncontrolled interactions and events, we deliberately sampled colonies with few contacts along their borders with adjacent animals, and with no evidence of disease, lesions, bare spaces, or other injury. Pieces of corals were broken off *in situ* using a chisel. Specimens were kept immersed in seawater, and then fixed in Helly's fixative within two hours of collection. Fixed tissues were decalcified using a solution of dilute HCl and EDTA and stored in 70% ethanol (Peters, 1984). Specimens were subsequently embedded in Paraplast (Sherwood Medical) and sectioned at 6 $\mu$m. The resulting slides were stained either with hematoxylin and eosin or with Heidenhain's Aniline Blue Method (Luna, 1968). Three slides were made at different depths within each specimen, one each from the top, middle, and bottom thirds of the tissues. Longitudinal sections of polyps were also prepared when possible.

On each cross-section of tissues, the abundance and condition of eggs, spermaries, and developing larvae were quantified by surveying 10 separate, non-overlapping fields at 125× magnification. Fields contained three to four polyps ($\bar{x} = 3.7$, S.D. = .71, no. fields counted = 79). Developmental stages of gonads and larvae were estimated by size and morphology (Fig. 1A-F, I). Statistical comparisons between central and peripheral tissues included data from slides cut from mid-depth in the tissues. All other statistical analyses (Sokal and Rohlf, 1969; Steele and Torrie, 1960) were performed on data from the slide of each specimen that revealed the maximum number of gonads.

### *Growth rate*

Growth rates for *P. astreoides* colonies were determined by staining colonies *in situ* with the vital stain Alizarin red S (*e.g.,* Dodge *et al.,* 1984). Alizarin red S is incorporated into a thin layer of skeleton during staining and provides a marker for measuring subsequent skeletal growth. In May of 1981, 10 colonies between 6 and 300 cm$^2$ were enclosed for 24 h in clear plastic bags containing a small amount of stain. Stained corals appeared to be healthy and lacked any evidence of injury or contacts with other sessile animals along their borders. After one year of growth, the colonies were collected and cut in half. Vertical growth was measured at five random locations along the cut face, and lateral growth was measured where five randomly chosen radii intersected the colony perimeter.

## RESULTS

Each factor examined—season, lunar day, polyp location, colony size, and colony age—influenced some aspect of reproduction or growth in *Porites astreoides.* The resulting patterns are complex; therefore, we have summarized the major findings of the results in Table I.

### *Breeding system*

*Gender and reproductive state of colonies.* Colonies of *P. astreoides* were either female or hermaphroditic. Of the 100 colonies sampled, 28% contained only female gonads, 26% contained both male and female gonads, and 43% contained no gonads. The only colony having exclusively male gonads contained only a single spermary. Thus, the existence of unisexual males in this population is doubtful. Brooded larvae were observed in 50% of female colonies and in 46% of hermaphroditic colonies.

*Distribution of gender among and within polyps.* Within hermaphroditic colonies, individual polyps could be either male, female, or hermaphroditic. The proportions of these three polyp types varied among colonies. Within hermaphroditic polyps,

TABLE I

*Summary of results*

A. BREEDING SYSTEM
  1. *Gender:* 52% female, 48% hermaphroditic
  2. *Fertilization:* Internal; larvae brooded

B. TIMING OF REPRODUCTION
  1. *Seasonal:* Peaks during April
  2. *Lunar:* Spermaries spawned around time of full moon; larvae mature prior to new moon

C. FACTORS AFFECTING POLYP FECUNDITY
  1. *Location within a colony:* Centers more fecund than colony edges
  2. *Colony size:* Associated with onset of female reproduction
  3. *Colony age:* Correlated with polyp fecundity in females

D. FACTORS AFFECTING COLONY GROWTH
  1. *Colony size:* Correlated with vertical and lateral growth rates

male and female gonads were occasionally found on the same mesentery (Fig. 1D, E). In both female and hermaphroditic colonies, gonads did not appear restricted to any particular mesenteries, although they were most frequent on incomplete mesenteries (Fig. 1H). Up to nine mesenteries might contain gonads within any given polyp.

Cross and longitudinal sections of mesenteries yielded similar counts of one or, rarely, two to three eggs per mesentery. In contrast, cross-sectional counts of spermaries could underestimate actual spermary abundance. Mesenteries in cross-section contained one to three spermaries. However, up to 14 spermaries, arranged vertically and occasionally overlapping, might be visible in longitudinal sections of mesenteries (Fig. 1E). There was no pattern to the vertical distribution of male and female gonads within hermaphroditic mesenteries (Fig. 1E).

### *Location of gonads within colonies*

*Centers* versus *edges.* Gonads were not distributed evenly throughout colonies. Comparison of central and peripheral tissues showed that the densities per polyp of both eggs and spermaries were greatest at colony centers (one-tailed, paired *t*-tests: For eggs, $P = .005$; for spermaries, $P = .016$). Brooded larvae were too rare to perform a statistical test of the relationship between abundance and position within colonies, but appeared to follow the same trend.

*Within centers.* The distribution of gonads among central polyps was patchy. In reproductive colonies, on average only half (51%) of the 10 fields surveyed in each central slide contained gonads (no. slides surveyed = 153).

*Within edges.* Due to the low density of gonads at colony edges, gender might easily be misidentified from samples of only peripheral polyps. Sixty-one percent of hermaphroditic colonies lacked male, female, or both types of gonads in peripheral polyps. Similarly, 36% of female colonies lacked eggs in peripheral regions. Only one of the 54 reproductive colonies contained gonads in peripheral tissues of a type (male) that was absent in the central tissues.

### *Development of gametes and larvae*

Female and male gamete development were divided into three approximate stages (early, middle, and late; Fig. 1).

*Female.* The earliest recognized female gametes had a basophilic cytoplasm and

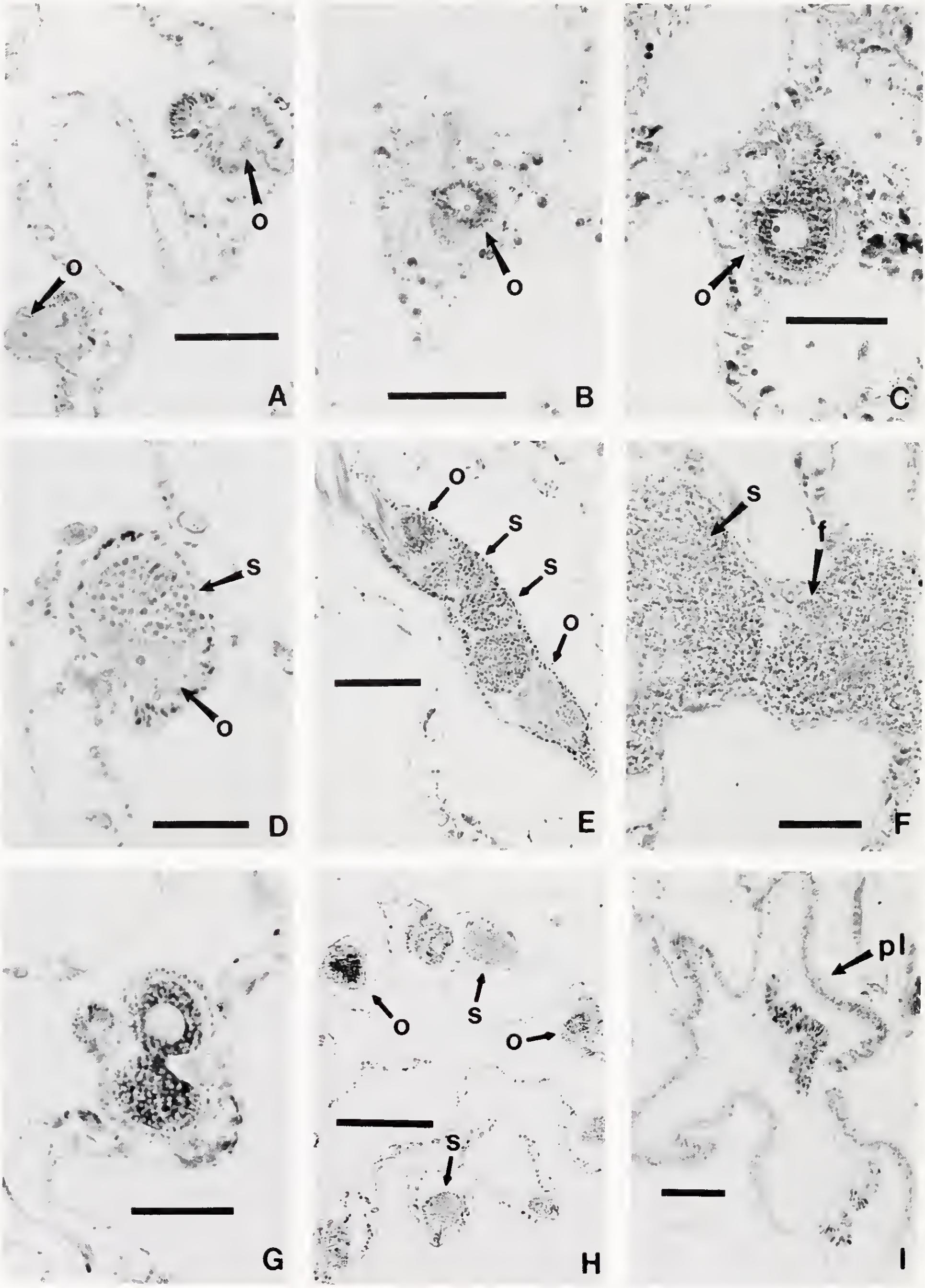

FIGURE 1. Gonad and larval development in *Porites astreoides*. A. Early female gametes (o) developing in two mesenteries. B. Later in development, the cytoplasm of oocytes changes in staining characteristics, and lipid droplets accumulate. C. Mature egg (o). Diameters of released eggs were between 130 and 170 $\mu$m in histological sections. D. Rare view of early oocyte (o) and spermary (s) developing on the same

an enlarged nucleolus. Initially present in the gastrodermis, the oocytes were later enveloped in the mesoglea (Fig. 1A). Mid-stage oocytes were larger and eosinophilic, with lipid droplets in the yolk (Fig. 1B). They had a distinctive eccentric nucleus and prominent nucleolus, and were surrounded by a thin layer of mesoglea. Mature eggs had completed vitellogenesis (Fig. 1C). Occasionally, eggs assumed a "dumbell" shape, with the nucleus in one lobe (Fig. 1G). Serial sections suggested that this unusual shape was a fixation artifact (but see Szmant-Froelich *et al.*, 1985).

*Male.* Male gametes developed from rounded interstitial cells with a large nucleus and little cytoplasm. These cells were found in the gastrodermis, although early spermatogonia were surrounded by mesoglea (Fig. 1D). Mid-stage spermaries were distinguished by the enlargement of irregularly shaped nests of cells with occasional mitotic figures visible (Fig. 1E). Later spermaries contained smaller spherical secondary spermatocytes and spermatids. Mature follicles were quite large, and stretched the mesenteries so that they were covered by only a layer of squammous gastrodermal cells and associated mesoglea (Fig. 1F). Mature spermatozoa with eosinophilic tails appeared first in follicle centers and eventually filled them completely. Spermatozoa appeared to burst from mesenteries, and large masses were found in polyp coelenterons and gastrovascular canals. Remnants of spermatozoa occurred in some mesenteries following spawning, occasionally accompanied by redeveloping spermaries.

*Larvae.* Fertilization for *P. astreoides* is internal. Mature eggs were observed being extruded between gastrodermal cells in mesenteries, and masses of sperm were seen surrounding released eggs in the coelenterons of several colonies. While most embryos were found in polyp coelenterons and gastrovascular canals, one appeared to lie within a mesentery, as observed by N. I. Goreau in Caribbean *Porites* spp. (Fadlallah, 1983). Early developing embryos consisted of numerous round blastomeres with external red granular yolky cells (Heidenhain's-stained) and a vacuolated central area. Zooxanthellae apparently invaded the endoderm of embryos as tissue layers differentiated. In later embryos, the interior was filled with foamy endodermal cells, and chromophore (lipofuchsintype pigment) cells and nematocysts appeared in the ectoderm. The most mature brooded planulae had six mesenteries, a well-formed stomodaeum, and embryonic chromophore cells and nematocysts in both the ectoderm and endoderm (Fig. 1I).

### *The timing of reproduction*

Reproductive events for *P. astreoides* occurred with both seasonal and lunar periodicities. The following analyses of reproductive patterns include only data from colony centers, since they provide the most reliable estimate of colony gender and fecundity.

*Seasonal periodicity.* Although gonads and larvae were present in at least some

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mesentery. Primary spermatogonia were 4–6 μm in diameter. E. Longitudinal section of mesentery containing two oocytes and six spermaries. F. Mature spermaries (s), as tails on spermatozoa begin to appear. A new follicle (f) is also beginning to develop. Individual spermatozoa had 1.5–2 μm triangular-shaped heads. G. "Dumbbell" shaped egg in mesentery. H. Cross-section through a polyp showing arrangement of eggs (o) and spermaries (s). I. Section through mature planula (pl) with six mesenteries and early filament tissues. Scale bars in A, B, C, E, F, and G, all equal 100 μm. Scale bar in D = 50 μm, and in H and I = 200 μm. Photomicrographs were taken using a Zeiss Photomicroscope II, and eggs and sperm were measured using a calibrated ocular micrometer under an oil immersion objective.

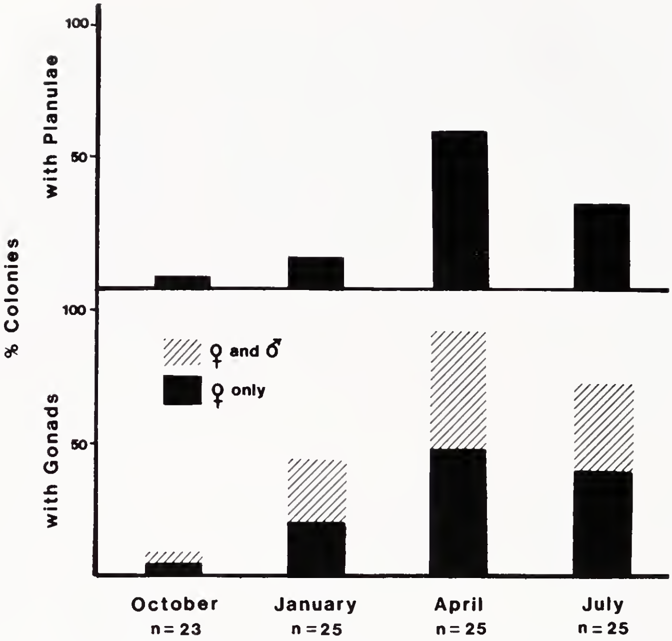

FIGURE 2. Seasonal variation in the proportion of colonies containing gonads or planula larvae.

colonies throughout the year (Fig. 2), there was a clear association between season and the numbers of colonies containing either gonads ($\chi^2 = 37.6$, df = 3, $P < .005$) or developing larvae ($\chi^2 = 22.67$, df = 3, $P < .005$). The maximum number of reproductive colonies occurred during April. Due to the low reproductive activity in October (2 colonies), analyses of reproductive patterns that follow compare only January, April, and July.

Among reproductive colonies, polyp fecundity varied seasonally (Table II). For both female and hermaphroditic colonies, the density of eggs in tissues differed among sampling months (Kruskal-Wallis comparisons: For females—H = 6.82, df = 2, $P < .05$; for hermaphrodites—H = 6.65, df = 2, $P < .05$). In particular, more ova were observed in April samples than in July (two-tailed, Mann-Whitney: For females—U = 97, n1 = 12, n2 = 10, $P < .02$; for hermaphrodites—U = 73, n1 = 11, n2 = 8, $P < .02$). Similarly, numbers of developing larvae also varied among months (Kruskal Wallis H = 80.4, df = 2, $P < .005$), with the highest values occurring during April. Interestingly, the numbers of male gonads in hermaphroditic colonies did not appear to vary seasonally (Kruskal-Wallis: H = 3.55, df = 2, $P > .1$).

*Lunar periodicity.* Superimposed upon the seasonal changes in reproduction was a pronounced lunar periodicity (Fig. 3). Most spermaries were mature prior to the full moon, and immature spermaries were most common between the full and new moons (Fig. 3A). In contrast, egg development showed no clear lunar pattern (Fig. 3B). Male and female gonadal abundance did not vary predictably with lunar day. Both the abundance (Fig. 3C) and maturity of brooded larvae peaked prior to the new moon.

TABLE II

*Seasonal variation in the density of gonads and developing larvae in tissues of* Porites astreoides*

| | Female colonies | | Hermaphroditic colonies | | |
|---|---|---|---|---|---|
| | Eggs | Larvae | Eggs | Spermaries | Larvae |
| Oct.** | .08 | 0.0 | .03 | .43 | 0.0 |
| Jan. | .40<br>(.22–1.7) | 0.0<br>(0.0–.03) | .57<br>(.08–1.9) | .20<br>(.05–.54) | 0.0<br>(0.0–.08) |
| April | .51<br>(.05–2.3) | .07<br>(0.0–.43) | .38<br>(.14–.78) | .20<br>(.05–1.5) | .03<br>(0.0–.62) |
| July | .18<br>(.03–.59) | 0.0<br>(0.0–.03) | .09<br>(.03–.54) | .07<br>(.03–.73) | .01<br>(0.0–.14) |

* Values presented as median number observed per polyp and the (range).

** No ranges given since only one female and one hermaphroditic colony were observed during October.

### *Differences among colonies*

*Gender.* Reproductive state was significantly associated with colony size for corals sampled during January, April, and July ($\chi^2 = 13.25$, df = 4, $P < .01$) (Fig. 4). Although the proportion of hermaphroditic colonies remained constant for all size classes, the proportion of non-reproductive colonies declined and the proportion of female colonies increased with increasing size class (Fig. 4). These trends suggest that hermaphroditic colonies are reproductive at relatively small sizes, whereas small colonies lacking gonads are females that will become reproductive when they increase in size. The presence of both females and hermaphrodites among the smallest colonies sampled (7 cm diameter, 38 $cm^2$), however, suggests that some unknown factor(s), in addition to size, may also influence the onset or continuation of female reproduction. Gender and reproductive state were not significantly associated with colony age (thickness).

*Fecundity.* Mean polyp fecundity differed widely among colonies (Table II, range values). To examine the potential sources for this variation, we compared the fecundity of all colonies of known size and age (thickness) from the April collecting period. This subsample was chosen because: (1) April was the month when the maximum number of colonies was reproductive and reproductive colonies were the most fecund; (2) gonadal abundance did not vary over a lunar period, allowing comparison among colonies sampled on different lunar days; and (3) previous studies have suggested that colony size (Kojis and Quinn, 1981; Babcock, 1984) and/or colony age (Rinkevich and Loya, 1979b; Kojis and Quinn, 1985) may influence the fecundity of coral polyps.

The potential influences of colony size and age (thickness) on fecundity were separated statistically by calculating partial correlation coefficients (Steele and Torrie, 1960). This is equivalent to calculating the correlation between fecundity and size while holding age constant (fs·a), and calculating the correlation between fecundity and age while holding size constant (fa·s).

Female colonies showed no significant correlation between fecundity and colony size ($r_{fs \cdot a} = -.35$, n = 9, $P > .05$). In contrast, female fecundity was significantly correlated with colony age (thickness) ($r_{fa \cdot s} = .91$, n = 9, $P < .01$), in part due to the

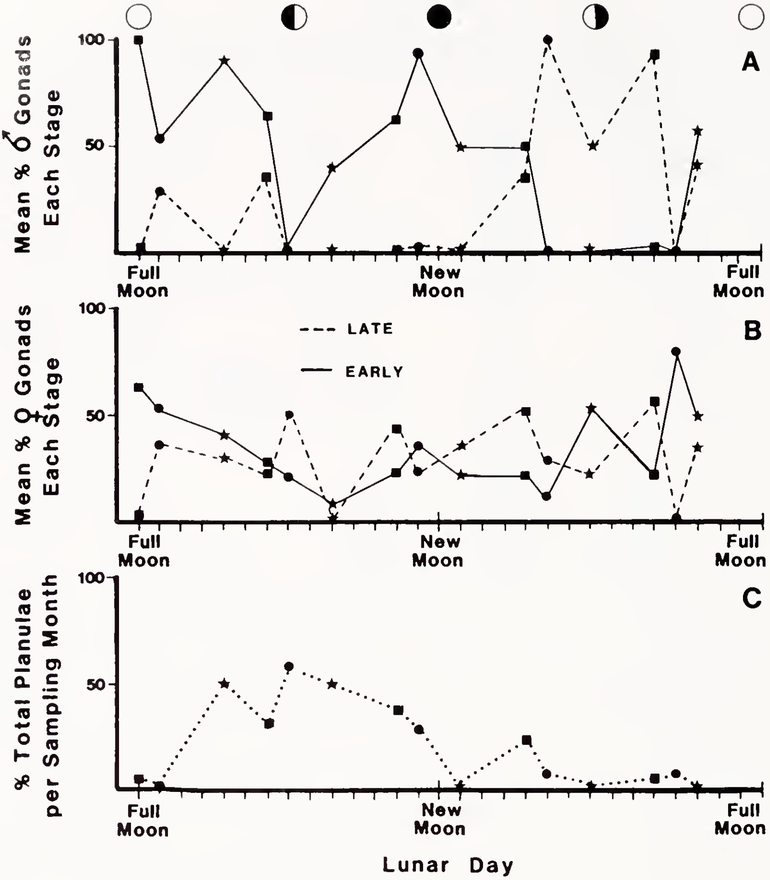

FIGURE 3. Lunar periodicity of reproduction in *Porites astreoides*. A. Lunar changes in the frequency of early (solid line) and late (dashed line) male gonads. Mature spermaries were most abundant prior to the full moon, and immature spermaries were most abundant between the full and new moons. B. Frequencies of immature (solid line) and mature (dashed line) eggs *versus* lunar day. There was no clear lunar pattern in the development of eggs. In both A and B, data are reported as the mean percent per colony of each stage. C. Most of the larvae observed were in tissues collected between the full and new moons. Figure presents data for January (stars), April (squares), and July (dots) sampling periods.

occurrence of a highly fecund small (212 $cm^2$) but old (6.5 cm thick) colony within the sample population (Fig. 5).

For hermaphroditic colonies, none of the partial correlations between either colony size or colony age (thickness) and any of three measures of fecundity—eggs per polyp, spermaries per polyp, or (eggs + spermaries) per polyp—was significant (for all six partial correlations, $.45 > r > -.51$, $P > .05$, $n = 9$). Similarly, the ratio of male:female gonads within hermaphroditic colonies (i) was not significantly correlated with either colony size ($r_{is \cdot a} = -.31$, $P > .05$) or colony age (thickness) ($r_{ia \cdot s} = .45$, $P > .05$).

### *Growth rate*

We examined two measures of growth: Lateral extension of the growing edge and vertical deposition of new skeletal material over the pre-existing skeleton (Fig. 6). The

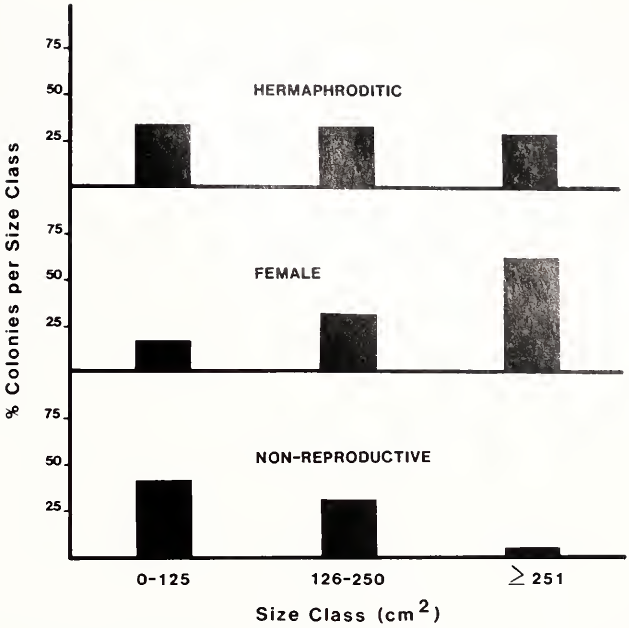

FIGURE 4. The relationship between gender and colony size. Colonies smaller than 125 $cm^2$ in surface area were mostly non-reproductive or hermaphroditic. With increasing size, the proportion of female colonies increased while that of non-reproductive colonies decreased. The frequency of hermaphroditic colonies remained approximately equal for all three size classes.

median rate of lateral growth was .73 cm/year (range = .2–1.8). The median rate of vertical growth was .31 cm/year (range = .13–.46).

Partial correlation coefficients were calculated to separate the potential influences of colony size and colony age (thickness) on growth rates. Rates of both vertical (v)

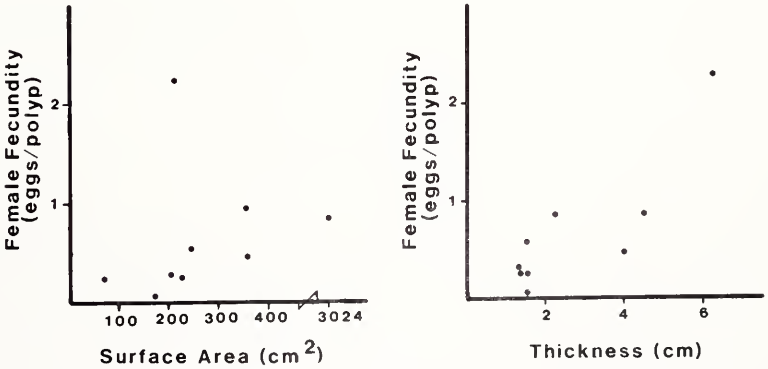

FIGURE 5. Female fecundity *versus* colony size and colony age. The fecundity of polyps in female colonies was significantly correlated with colony thickness (an estimate of minimum colony age) but was not correlated with the surface area of live colony tissues (colony size).

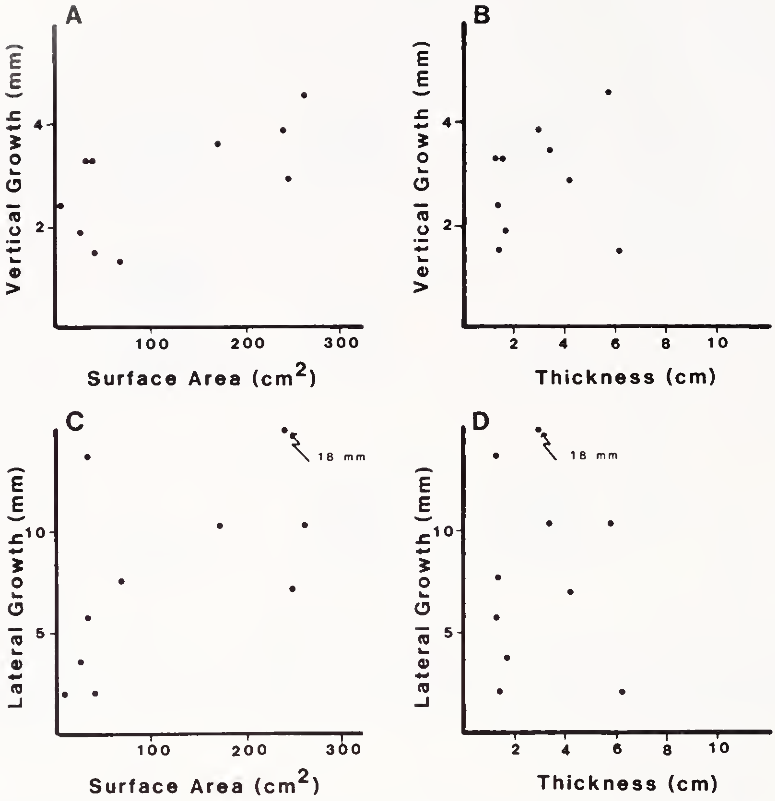

FIGURE 6. Colony growth *versus* colony size (surface area) and colony age (thickness). Vertical and lateral growth were both significantly correlated with colony surface area (A, C) and not with colony thickness (B, D).

and lateral (l) growth were significantly correlated with colony size ($r_{vs \cdot a} = .75$, $r_{ls \cdot a} = .67$, $P < .05$) (Fig. 6A, C). In contrast, partial correlations between vertical or lateral growth rate and colony age (thickness) were clearly not significant ($r_{va \cdot s} = -.3$, $r_{la \cdot s} = -.04$, $P > .05$) (Fig. 6B, D).

## DISCUSSION

### *Periodicity of sexual reproduction*

Sexual reproduction for the abundant Caribbean reef coral *Porites astreoides* occurs with both seasonal and lunar periodicities in Jamaica. Although some colonies contained gonads throughout the year, the proportion of reproductive colonies in the population and the fecundity of coral colonies varied seasonally. Colonies were maximally reproductive during the spring (April). Superimposed upon this annual pattern was a synchronized, lunar periodicity in the development of male gonads and larvae. Our data suggest male gametes are spawned prior to the full moon, and larvae are released around the time of the new moon. The lack of any clear lunar pattern in the development of female gonads suggests that eggs may take more than a month to mature and/or may vary widely in developmental rate.

The subtle seasonal changes in coral reproduction described here (and elsewhere for other corals, *e.g.*, Harriot, 1983; Kojis and Quinn, 1984; Stoddart and Black, 1985) may have important implications for evaluating the annual reproductive activity of individual colonies. For example, variation among months in the frequency of reproductive colonies (Fig. 2) suggests that individual corals within a population may differ in the duration of their annual reproductive periods. In addition, seasonal differences in coral fecundity suggest that the fecundity of any given coral colony may vary throughout the year. Clearly, estimates of reproductive output based on observations at any single time of year or of only a few colonies would be misleading for this and, perhaps, other corals.

### *Breeding system*

*P. astreoides* colonies are either female or hermaphroditic. This is the first description of a mixed breeding system of this type for corals—most appear to have predominantly unisexual (dioecious) or hermaphroditic (monoecious) colonies within a population (for reviews see Fadlallah, 1983; Harrison, 1985). It differs from Szmant's (1986) description of *P. astreoides* from Puerto Rico as simultaneously hermaphroditic, and is also in striking contrast to Indo-Pacific species of *Porites* that appear to be largely dioecious (Kojis and Quinn, 1982; Harriott, 1983; Harrison, 1985; Szmant, 1986) [although Kojis and Quinn (1982) did report a very low incidence of hermaphroditic colonies in the Pacific *P. andrewsi*].

The simultaneous occurrence of female and hermaphroditic colonies within a population might simply indicate that all colonies are potentially hermaphroditic, but male gonads have been missed in apparently female colonies due to a sampling error. However, the low probability of gender being misidentified from central tissues (1/54) suggests that *P. astreoides* having only female gonads within centrally located polyps contain only female gonads throughout the colony.

Alternately, female colonies might occur within a hermaphroditic population if gender varies seasonally or over a coral's lifespan, and, consequently, corals produce male gametes for only part of the time that they are reproductive (*e.g.*, Fadlallah, 1983). The temporally constant sex ratio in this population of *P. astreoides* (Fig. 2), however, suggests that seasonal shifts in gender are unlikely. Similarly, there does not appear to be a predictable change in gender associated with colony size and/or age (Rinkevich and Loya, 1979a, b; Kojis and Quinn, 1981). If colonies did switch from hermaphroditic to female with increasing size, the relative frequency of hermaphrodites would decline in larger size classes and the ratio of male to female gonads within hermaphroditic colonies should be negatively correlated with colony size. Neither are true for *P. astreoides.* Gender also does not appear to be a function of aging for *P. astreoides,* as both gender and the ratio of male to female gonads within hermaphroditic colonies were not associated with our estimate of colony age (thickness).

The combined results suggest, instead, that colonies of *P. astreoides* are either female or hermaphroditic and that hermaphroditic colonies are generally reproductive at smaller sizes than females (Fig. 4).

The apparent lack of true males within this population of *P. astreoides* suggests that there may be a reproductive disadvantage associated with making only male gametes (*e.g.*, Charnov, 1982). That is, individuals investing reproductive resources exclusively into male gonads would be less successful at producing offspring than hermaphrodites. A mixed breeding system like that of *P. astreoides* might be selected for, for example, if the likelihood that sperm will fertilize eggs of another colony is unpredictable or temporally variable.

The unusual breeding system of *P. astreoides* also has important genetic implications. Some hermaphroditic corals are capable of self-fertilization (Kojis and Quinn, 1981; Heyward and Babcock, 1986). While cross-fertilization must occur in female colonies of *P. astreoides,* the proximity in hermaphroditic colonies of eggs and spermaries on some mesenteries suggests the potential for self-fertilization in this gender. If females and hermaphrodites of *P. astreoides* do differ in mode of fertilization, their respective offspring will also differ in genetic composition and diversity.

### *Ontogenetic changes and energetic constraints*

Several life history features of *P. astreoides* appear to be under independent control; some are associated with colony size and others are associated with colony age (thickness). Moreover, it seems that the influence of at least colony size may differ between genders, as reflected by the difference between female and hermaphroditic colonies in the size at which most colonies become reproductive (Fig. 4).

When life history features of colonial animals, such as sexual maturity, are dependent upon colony size, they may vary as colonies grow or are reduced in size by external events (*e.g.,* Wahle, 1983, 1985; Hughes and Jackson, 1985; Kojis and Quinn, 1985; Szmant-Froelich, 1985; Karlson, 1986). For *P. astreoides,* reproductive state, vertical growth rate, and lateral growth rate are associated with colony size. Thus, these attributes potentially may change over a coral's lifespan as colony size increases or decreases.

Sexual reproduction and growth are often described as competing biological functions that draw on a limited supply of energetic or material resources within the individual (*e.g.,* Stearns, 1977). It has been predicted that rates of growth in colonial animals should decrease following the onset of sexual reproduction (Williams, 1975; Jackson, 1979; Kojis and Quinn, 1981). However, for *P. astreoides,* growth rates increase over the range of colony sizes associated with the onset of female reproduction (compare Figs. 4 and 6), suggesting that the presumed costs of gametogenesis do not result in a measurable depression of growth.

For female colonies of *P. astreoides,* polyp fecundity is associated with colony age (thickness) and not with colony size (Fig. 5). This suggests that polyp fecundity is a function of colony aging (*e.g.,* Kojis and Quinn, 1985). Thus total colony fecundity will be related both to the number of reproductive polyps within a colony and to colony age. The former often may be determined largely by colony size.

The lack of any correlation between fecundity and either size or age (thickness) among hermaphroditic colonies may reflect the difficulty of quantifying hermaphrodite fecundity. For example, there are different sampling biases inherent in estimates of egg *versus* spermary abundance from polyp cross-sections—because eggs and spermaries differ both in their distributions within mesenteries and in their potential residence times within coral polyps. Moreover, spermaries and eggs probably constitute quantitatively different investments of colony resources, contribute differently to the successful production of offspring, and thus cannot be compared directly. Such considerations will be important for accurately ranking or interpreting the fecundity of hermaphroditic colonies having different relative proportions of female and male gonads.

Patterns of gonad and larval abundance within individual colonies of *P. astreoides* may reflect energetic and/or ontogenetic constraints. Low densities at colony edges might occur because the proximate allocation of colony resources to growth along the edge reduces the local resources available for gamete production (*e.g.,* Williams, 1975). Alternatively, lower fecundity along the edges of colonies might be an evolved "strategy" to ensure that valuable gonads are not located in colony tissues most likely to experience injury due to competition, predation, or growth into unsuitable habitats (*e.g.,* Jackson, 1979). Finally, if polyp fecundity is influenced by polyp age (Fadlallah, 1982; Wallace, 1985), higher fecundity in central polyps might simply reflect their relative age and stability. Although these three hypotheses are not mutually exclusive, we favor the last for *P. astreoides,* since it is consistent with the positive correlation observed between the fecundity of female polyps and colony age (thickness).

### *Conclusion*

Patterns of sexual reproduction and growth described here for *P. astreoides* are complicated by the interaction of the following factors: (1) Temporal patterns of reproduction; (2) "pre-programmed" differences among colonies in fecundity apparently associated with aging; (3) size-related variation among colonies in reproductive condition and growth rate; and (4) differences between colony regions in fecundity. In large part, this complexity is related to the colonial mode of construction which allows reproductive activity to differ among polyps of a colony, and the life history features associated with size and age to vary independently.

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# THE FORMATION OF CLONAL TERRITORIES IN EXPERIMENTAL POPULATIONS OF THE SEA ANEMONE *ACTINIA TENEBROSA*

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## ABSTRACT

Fine and gross-scale clumping of electrophoretically identical anemones (genotypic clumping) was detected 11 months after multi-clonal groups of adults had been transplanted into rock pools on two shores. The locomotory separation of non-clonemates following conflicts appeared to be the primary cause of gross-scale genotypic clumping of adults, and the fine-scale genotypic clumping of adults and recruits. The gross-scale clumping of adults and recruits must reflect the effects of localized asexual recruitment. In contrast, genotypic clumping was not detected within a third population with lower recruitment, located on a relatively smooth shore. In that population, adults were typically restricted to small individual depressions and movement was rarely detected. These data support the hypothesis that intergenotypic aggression may play an important role in determining the genotypic structure of populations, and indicate that the importance of this factor may be partially determined by the topography of the shore.

## INTRODUCTION

Within several species of sea anemones, interclonal aggression is thought to play a major role in the formation of clonal aggregations (Francis, 1973a, b; Purcell, 1977; Sebens, 1982; Ayre, 1983a). Laboratory trials (Francis, 1973b; Ayre, 1982) and field observations (Ottaway, 1978) have shown that intraspecific aggression leads to the locomotory separation of adult anemones. Nevertheless, the importance of aggression in the field has chiefly been inferred from observations of seemingly static clonal boundaries. The most striking examples have been documented for the fissiparous *Anthopleura elegantissima,* where anemone-free zones have remained between clones for periods of up to four years (Francis, 1973a). However, in these cases it has not been possible to separate the effects of aggression from those of other factors, such as passive growth and dispersal, which may determine clonal distributions. Furthermore, the role of aggression has been obscured by recent reports that aggressiveness may be reduced or absent in pairs of non-clonemate *Metridium senile* of different sex (Kaplan, 1983) and for pairs of non-clonemate *M. senile* (Purcell and Kitting, 1982) and *Anthopleura xanthogrammica* (Sebens, 1984) within genetically diverse aggregations.

*Actinia tenebrosa* is an asexually viviparous, solitary species which is also believed to reproduce sexually, producing widely dispersed, genetically diverse colonists (Black and Johnson, 1979; Ayre, 1983a; 1984a). *A. tenebrosa* is dioecious (Ottaway, 1979) and it appears likely that each clone is unisexual (Ayre, 1984c). The majority of recruits into natural populations on stable rock platforms are the asexually produced

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juveniles of resident adults (Black and Johnson, 1979; Ayre, 1983a). Brooded juveniles may settle at least several meters from their brood parents and consequently, local populations typically consist of at least several intermingled clones (Ayre, 1983a, 1984a, b). Nevertheless, clones may have restricted (clumped) distributions and it has been suggested that interclonal aggression is used to maintain and extend clonal territories (Ayre, 1983a). Adult *A. tenebrosa* fight using specialized nematocyst-bearing structures called acrorhagi (Ottaway, 1978). These can be used to attack adult or juvenile non-clonemates within tentacle contact (Ayre, 1982). Each fight may last for as little as a few minutes. In some cases acrorhagial scarring persists and is visible for several days. In the field, resident adults are most often able to repel adult immigrants (Ottaway, 1978). In the laboratory, even mild attacks may significantly increase the level of juvenile mortality (Ayre, 1982).

This paper documents the active behavioral formation of clumps of genotypically identical anemones within experimental populations of *A. tenebrosa* and provides an indirect test of the hypothesis that intergenotypic aggression is directed only against non-clonemates of the same sex.

## MATERIALS AND METHODS

### *Experimental design*

All experiments were conducted on exposed, sloping limestone shores within three natural populations of *A. tenebrosa* on the coast of Rottnest Island, Western Australia (32°S; 115°30E). All adult anemones ($\geq 10$ mm column diameter; Ayre, 1984b) were found within a band of shore approximately 1 m high, in the lower and mid-intertidal zone. The experimental sites were on shores of two topographic types. Two sites were within areas of small rockpools on shores at Green Island and Salmon Point. Pools ranged up to 70 cm in diameter and some individual pools contained more than 50 adults. The third site was an area of smooth shore within one of three populations at Strickland Bay (Ayre, 1983b). These adults were typically isolated within small depressions (2 to 3 cm diameter).

To test the hypothesis that the formation of clumps of genotypically identical adult *A. tenebrosa* has a behavioral basis, multi-clonal groups of 40 adults were haphazardly transplanted onto each of twenty-seven 1-m long strips of shore. This was a reciprocal transplantation experiment in which adults were transplanted within and between the three populations (Ayre, 1985). Three study sites were established within each population, and at each site a 1-m strip of shore was allocated to anemones from each of the three populations. Transplants were performed in early November 1980, following the removal of all resident anemones from each of the 27 transplant strips. All adults were transplanted into the zone from which anemones had been removed, but were never intentionally returned to their original positions. All transplanted adults rapidly attached themselves to the limestone shore and all were in place for at least one hour prior to exposure to wave action. Detailed topographic maps, based on sketches and photographs, were prepared for each strip. To ensure that the transplanted adults could be distinguished from recruits that had grown to $>10$ mm (new adults), the locations and sizes of all transplanted adults, new adults, and juveniles were monitored at approximately two-month intervals. After 11 months the positions of all surviving adults and new recruits were recorded and all anemones were then collected. Adults were stored, live, at 4°C for up to five days prior to dissection, and adults and all juveniles were then stored at −20°C pending electrophoresis. The sexes of adults were determined on the basis of the color and morphology of their gonads (Ayre, 1984b).

### *Electrophoresis*

All anemones were assayed for the five polymorphic enzymes catalase (CAT, EC 1.11.1.6), malate dehydrogenase (MDH, EC 1.11.1.37), mannose phosphate isomerase (MPI, EC 5.3.1.8), phosphoglucomutase (PGM, EC 2.7.5.1), and superoxide dismutase (SOD, EC 1.15.1.1), as described by Ayre (1983a). Anemones were considered to be non-clonemates if they had different genotypes at one or more loci. Electrophoretically identical anemones might be either clonemates or non-clonemates.

### *Analysis*

Clumping of electrophoretically identical (genotypic clumping) anemones was examined on two spatial scales (Ayre, 1983a). Within each population, fine-scale genotypic clumping, on a scale consistent with the predicted effects of intergenotypic aggression, was tested by comparing the proportions of anemones with only genotypically identical neighbors within a two-cm radius (*i.e.*, within tentacle contact; Ayre, 1982) and a 2–4 cm radius. It was assumed that if aggression was occurring then a disproportionate number of near neighbors should be electrophoretically identical. Gross-scale genotypic clumping within each whole transplant strip, resulting from differential dispersal or mortality, was tested for by comparing the mean distances between all pairs of electrophoretically identical anemones and all pairs of electrophoretically different anemones. This analysis was conducted for each multi-locus genotype represented by two or more anemones. Genotypes were considered to be clumped if the mean distance between pairs of anemones with that genotype was less than the mean distance between them and all other anemones. These results were then pooled for all strips within each population, and the proportions of clumped and unclumped genotypes were compared with the expectation that half of all genotypes would have clumped distributions due to chance alone. These tests for genotypic clumping should be conservative since it may not be possible to distinguish all clones using only five variable loci and the areas of the transplant strips may be small relative to the limits of juvenile dispersal.

## RESULTS

### *Survival and recruitment*

At the conclusion of the experiments, the mean numbers ($\bar{x}$ ± S.E.) of surviving adults per strip were similar within each of the three populations ranging from 17 ± 2 at Strickland Bay and Salmon Point, to 21 ± 2 at Green Island. As was expected, all adults were restricted to the shelter of pools and depressions whereas many juveniles settled on exposed shore (Fig. 1). Settlement and recruitment varied considerably within and between colonies, and 445, 187, and 51 recruits were collected from Green Island, Salmon Point, and Strickland Bay, respectively.

### *Fine-scale genotypic clumping*

Genotypic clumping on a scale consistent with the predicted effects of intergenotypic aggression was detected on the rough shore within both the Green Island and Salmon Point populations (Table I). Disproportionately more anemones had only genotypically identical neighbors within a 2 cm radius compared with the proportions within a 2- to 4-cm radius. Significant levels of fine-scale clumping were detected within the groups of transplanted adults collected from Salmon Point and within the groups of transplanted adults and recruits collected from both Green Island and

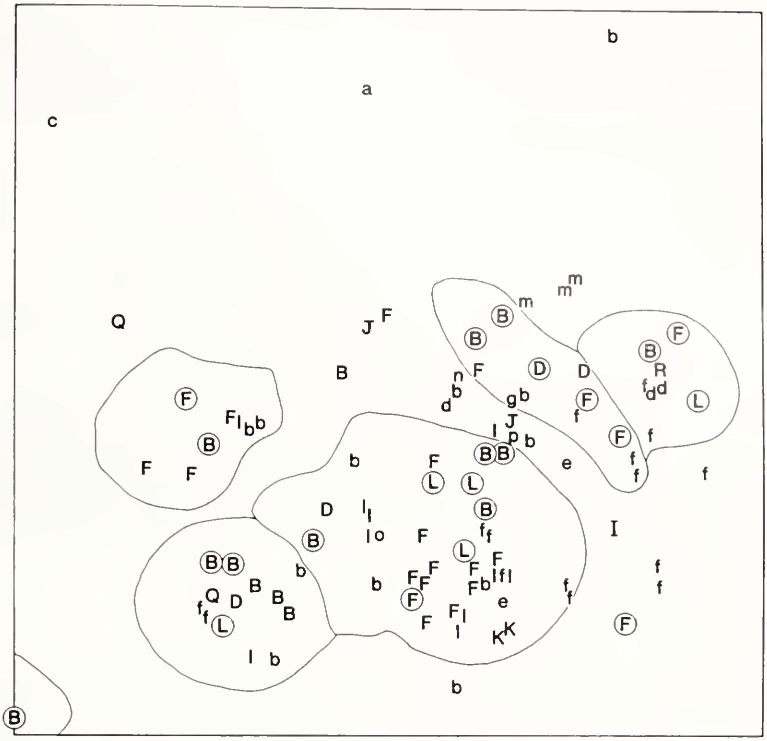

FIGURE 1. The distribution of all adult and juvenile *Actinia tenebrosa* 11 months after a multi-clonal group of 40 adults had been transplanted haphazardly into rock pools (outlines shown as lines) on a 1-meter wide strip of shore at Green Island. Each anemone is represented by a letter denoting its size and age, and 5-locus genotype. A total of 18 different genotyopes are represented. This strip was chosen at random from the set of 27 experimental sites. Transplanted adults = enclosed upper case letter; adult recruits = upper case letter; juvenile recruits = lower case letter.

Salmon Point (Table I). In contrast there was no such clumping of electrophoretically identical adults at Strickland Bay. In fact, a greater proportion of anemones had electrophoretically different neighbors within a 2-cm radius as compared with a 2- to 4-cm radius (Table I), although this difference was not statistically significant. There was some evidence of fine-scale genotypic clumping of transplanted adults and recruits at Strickland Bay, however these data provided only a weak test for such clumping as only 10 recruits were found within 4-cm of transplanted adults (Table I).

### *Gross-scale genotypic clumping*

Each of the 27 transplant strips was found to support anemones with at least 3, and typically 5 or more, 5-locus genotypes. Genotypes were intermingled on the shore, and many juveniles were detected outside the limits of adult distributions (Fig. 1). Nevertheless, for transplanted adults, a significant majority of genotypes were judged to be clumped within the Green Island and Salmon Point strips (Table II), although these results were not statistically significant for either individual population. In contrast only half of the 45 groups of genotypically identical adults on the smooth shore at Strickland Bay were judged to be clumped, the proportion expected

TABLE I

*Comparison of the numbers of adult* Actinia tenebrosa *with only genotypically identical adult neighbors*[a] *(S) or at least one genotypically different neighbor (D) within a 2-cm and 2 to 4-cm radius*

| | | Separation | | | | | |
|---|---|---|---|---|---|---|---|
| | | <2 cm | | 2 to 4 cm | | | |
| Population | Pairing | S | D | S | D | $\chi^2_{(1),1}$ | *P* |
| Green Island | Adult-adult | 18 | 12 | 17 | 28 | 2.74 | <0.05 |
| Green Island | Adult-recruit | 65 | 28 | 32 | 68 | 26.2 | <0.001 |
| Salmon Point | Adult-adult | 19 | 4 | 13 | 17 | 6.83 | <0.005 |
| Salmon Point | Adult-recruit | 14 | 13 | 5 | 21 | 7.62 | <0.005 |
| Strickland Bay | Adult-adult | 12 | 21 | 15 | 12 | 1.50 | N.S. |
| Strickland Bay | Adult-recruit | 5 | 0 | 2 | 3 | Fisher's Exact Test | 0.083 |

Comparisons are made for pairs of transplanted adults, and transplanted adults and adult and juvenile recruits. Data are presented for anemones within three experimental populations, which had been established 11 months earlier by haphazardly transplanting multi-clonal groups of 40 adult anemones onto cleared areas of shore.

[a] Based on 5 locus-genotype.

N.S. $P > 0.05$.

by chance alone (Table II). In addition, at Green Island, a significant majority of the groups of electrophoretically identical transplanted adults and recruits were judged to be clumped (Table II, $P < 0.001$). In contrast only half of such groups were clumped within the Salmon Point and Strickland Bay populations (Table II), however, this may reflect the underlying unclumped distribution of genotypes represented by transplanted adults. Furthermore, the present experimental design provides only a weak test for gross-scale genotypic clumping involving recruits since each test was restricted to only a 1-m$^2$ experimental strip. In natural populations juveniles may be dispersed passively over several meters and genotypic clumps are often spread over at least several square meters of shore (Ayre, 1983a). Therefore, this difference between populations may be a real environmental effect but cannot be considered evidence that the same processes do not operate on all three shores.

### *Effects of sex*

The distribution of the 59 pairs of electrophoretically distinct sexually mature adults separated by ≤4 cm did not support the hypothesis that aggression is directed only towards non-clonemates of the same sex. The proportions of electrophoretically different, same-sex and mixed-sex pairings separated by <2 cm and 2–4 cm were not significantly different (Table III). This implies that if intergenotypic aggression is a major determinant of the distribution of genotypes on these shores then the initiation of conflicts is not affected by similarity of sex.

## DISCUSSION

The rapid formation of genotypic clumps within experimental populations of adult *A. tenebrosa* supports the hypothesis that clonal distributions may be, at least partially, determined by the effects of interclonal aggression and subsequent migra-

TABLE II

*The incidence of gross genotypic*[a] *clumping within experimental groups of* Actinia tenebrosa *in each of three populations*

| Group | Population | Genotype | | | |
|---|---|---|---|---|---|
| | | Clumped | Not-clumped | $\chi^2_{(1),1}$ | |
| Transplanted adults | Green Island | 18 | 11 | N.S. | |
| | Salmon Point | 15 | 7 | N.S. | |
| | Strickland Bay | 23 | 22 | N.S. | |
| Transplanted adults and recruits | Green Island | 50 | 19 | 13.04 | <0.001 |
| | Salmon Point | 23 | 22 | N.S. | |
| | Strickland Bay | 12 | 14 | N.S. | |

Data are presented for adult anemones which had been haphazardly transplanted 11 months prior to sampling, and for those adults and all adult and juvenile recruits. Chi-squared analyses were used to determine if a significant majority of genotypes were clumped.

[a] For each 5-locus genotype, anemones were judged to be clumped if the mean distance between all anemones with that genotype was less than the mean distance between all anemones with different genotypes.

N.S. not significant $P > 0.05$.

tion (Ayre, 1983a). Similarly, genotypic clumping has been described within natural populations of *A. tenebrosa* (Ayre, 1983a) where interclonal conflicts have been observed. These findings, together with experimental demonstrations that these clones are locally adapted (Ayre, 1985), support the theoretical prediction that inter-clonal competition should restrict clones to those parts of the habitat to which they are best adapted (Williams, 1975).

In this study both fine and gross-scale genotypic clumping developed within at least some of the multi-clonal groups of adults which had been established 11 months earlier by haphazard transplantation. Genotypic clumping on both scales is most simply explained by the differential migration of genotypes following interclonal conflicts, or by the preferential association of clonemates. Fine-scale clumping of adults occurred on a scale that was consistent with the predicted, aggressive repulsion of non-clonemate near neighbors. Disproportionately more pairs of anemones within

TABLE III

*Comparison of the numbers of pairs of genotypically different*[1] *adult* Actinia tenebrosa *with the same or different sex which were separated by <2 cm and 2 to 4 cm*

| Pairings | Separation | | |
|---|---|---|---|
| | <2 cm | 2 to 4 cm | |
| Same sex | 8 | 15 | |
| N.S. | | | $\chi^2_{(1),1} = 0.32$ |
| Male-female | 10 | 26 | |

Results were pooled for groups of anemones which had been haphazardly transplanted into 3 populations 11 months prior to sampling.

[1] Based on 5 locus-genotype.

N.S. $P > 0.05$.

the range of tentacle contact were genotypically identical than those just outside the range of contact (2–4 cm). Such fine-scale genotypic clumping could also be explained by the differential migration of adult clonemates into favored micro-habitats, but this seems inconsistent with the overlapping gross-scale distribution of genotypes detected in this study (Fig. 1) and within natural populations (Ayre, 1983a). Gross-scale genotypic clumping on rough shores could have resulted from the aggressive repulsion and subsequent migration of non-clonemates, differential migration of genotypes into particular microhabitats, or differential mortality of clones within microhabitats. It is not possible to distinguish between the first two hypotheses. However, the third hypothesis is weakened by the fact that, although mortality rates were similar in each population, no evidence of gross genotypic clumping was detected at Strickland Bay. At Strickland Bay pedal locomotion is severely restricted by the topography of the shore, since anemones are almost all restricted to small, individual pools. As might be predicted, because adult anemones on that shore were rarely within tentacle contact, no intraspecific fighting was observed or acrorhagial scarring detected during a three-year study of both the experimental and natural populations at that location. In contrast, 12 fights between pairs of adults were inferred from the presence of acrorhagial scarring during the present 11-month study of experimental populations at Green Island and Salmon Point. In eight of these cases one adult in each pair died or was lost from the study site within the following two months, which may be compared with an average of 7% mortality per 2-month period for all adults in this experiment (Ayre, 1985).

At Green Island, statistically significant gross-scale genotypic clumping of transplanted adults and adult and juvenile recruits was superimposed upon the already clumped distributions of the groups of transplanted adult clonemates. This result strongly supports the prediction that, within natural populations, clonal territories are extended by the combined effects of interclonal aggression and the passive dispersal of asexually produced juveniles (Ayre, 1983a). Passive dispersal should always play a major role in extending clonal boundaries. However, the fine-scale clumping of adults and juveniles, detected at Green Island and Salmon Point, supports the hypothesis that this effect may be strengthened by intergenotypic aggression (Ayre, 1983a). Such fine-scale genotypic clumping of adults and juveniles should be expected to develop on all types of shore as a result of attacks on juvenile non-clonemates which settle close to adults. Nevertheless, it is clear from this study and earlier work (Ayre, 1985) that environmental factors affecting populations can greatly influence the rate of expansion of clones through effects on asexual fecundity, recruitment, and the frequency of intergenotypic conflicts.

The conclusions of this and earlier studies of the genetic structure of populations of *A. tenebrosa* (Black and Johnson, 1979; Ayre, 1983a, 1984a, b) are in apparent contrast with the outcome of studies of the northern hemisphere species *Actinia equina* var mesembryanthemum. Several authors have claimed that in British populations there is little fine or gross-scale clumping of clonal genotypes and that asexually produced juveniles are in fact widely dispersed (Orr *et al.,* 1982; Quicke and Brace, 1983; Brace and Quicke, 1985, 1986a, b). The latter conclusion is based principally on the finding that single locus genotype frequencies approach the values predicted for Hardy-Weinberg equilibria, an outcome which is more consistent with the recruitment of sexually reproduced recruits. In addition, Brace and Quicke (1986b) have concluded that for *A. equina* the principal role of intraspecific aggression is "to act to enhance adult survivorship through the securement of space . . ." Although Brace and Quicke (1985) emphasize the apparent differences between populations of the two species, they recognize that in their studies environmental differences between sites may account for differences in the importance of both localized asexual recruitment and inter-genotypic aggression. In fact they conclude that in the single area in which genotypic clumping was detected anemones may have been less susceptible to dislodgement resulting from wave action. It could also be argued that only two of their four study sites contained anemones at sufficiently high densities (<2.5 cm mean nearest neighbor distance) to provide a meaningful test of the effects of inter-genotypic aggression, and that at least three of their study sites were too small (0.18–0.59 $m^2$) to provide a useful test for gross-scale genotypic clumping. This apparent contrast of life-history tactics may therefore simply reflect the decision to study populations of *A. equina* in which low levels of asexual recruitment and interclonal aggression are favored, and the use of different methods of analysis in the Australian and British studies. It is certainly true that interpopulation variation with respect to many life-history characters has been documented for other anemones, including the contributions of sexual and asexual reproduction to recruitment, as inferred from allozyme data, for *A. tenebrosa* (Ayre, 1984a) and *Metridium senile* (Hoffmann, 1986).

The present results do not support the idea that the aggressive response of adult *A. tenebrosa* to adult non-clonemates (Ayre, 1982) is reduced when pairs of adults are of different sex. Similar proportions of same sex and mixed sex pairs were detected within and immediately beyond the range of tentacle contact. Since only 59 suitable pairings were recorded, it is possible that similarity of sex may have some influence on the aggressiveness of pairs. This finding contrasts with Kaplan's (1983) clear demonstration that mixed sex pairs of *Metridium senile* would not fight. Sebens (1984) also failed to find any evidence that the incidence of aggression is affected by similarity of sex in populations of *Anthopleura xanthogrammica.* These results, together with other reports of variation in the sensitivity of histocompatibility reactions in coelenterates (Resing and Ayre, 1985) and the finding that factors such as age (Hidaka, 1985) and prolonged contact with non-clonemates may modify their expression (Purcell and Kitting, 1982; Sebens, 1984), highlight our limited understanding of these processes.

Although the sensitivity of the histocompatibility response has not been thoroughly tested for *A. tenebrosa,* it is clear that intergenotypic aggression plays an important role in determining the genotypic structure of populations of *A. tenebrosa* on some shores, where it must therefore play an important role in inter-clonal competition. The finding that genotypic clumping did not develop on the smooth shore at Strickland Bay implies that interclonal aggression accelerates and enhances the effects of passive asexual dispersal on the development of clonal territories. The reciprocal transplant study which generated the data described in the present study revealed that clones of this species were highly locally adapted. With regard to asexual fecundity, however, the importance of inter-genotypic aggression as a component of clonal fitness has not been determined. It seems likely that aggressiveness should be most strongly selected for on shores which support moderate or high densities of mobile adults.

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# THE ALLOMETRY OF DEPOSIT FEEDING IN *CAPITELLA* SPECIES I (POLYCHAETA:CAPITELLIDAE): THE ROLE OF TEMPERATURE AND PELLET WEIGHT IN THE CONTROL OF EGESTION

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## ABSTRACT

This study investigates the relationships between egestion rate, body size, and environmental temperature in the opportunistic marine polychaete *Capitella* species I. Measurements were made of (1) fecal pellet weight, (2) pellet production rate, and (3) pellet standing stock within the gut of live worms. Egestion rate experiments were conducted with worms ranging in size from 0.27 $mm^3$ to 2.62 $mm^3$ (approx. 1.0–15 mm in length). Pellet production rate measurements were made at 15°, 20°, and 25°C. Individual fecal pellet weight was related to worm body volume to the 0.70 power. Fecal pellet production rate in *Capitella* sp. I was independent of body size. The number of pellets maintained on average within the guts of larger animals is at least equal to that of smaller animals. Fecal pellet production rate increased exponentially with increasing temperature between 15°C and 25°C, with an overall $Q_{10}$ value of 2.49. Power functions relating changes in egestion rate ($\mu$g sediment $h^{-1}$) and size-specific egestion rate [$\mu$g dry weight sediment ($mm^3$ worm)$^{-1}$ $h^{-1}$] to body size were fit to the data. These curves show that egestion rate scales as body volume to the 0.70 power, indicating that larger *Capitella* sp. I specimens process relatively less sediment per unit body volume than smaller worms. *Capitella* sp. I individuals thus control sediment processing rate during ontogeny by reducing the relative pellet weight as they grow.

Estimates of the scaling of external surface area in *Capitella* sp. I show that surface area scales as worm size to the 0.77 power, paralleling the scaling of feeding rate to body size. Data on oxygen uptake rates at different temperatures for *Capitella* sp. I (Cammen, 1985) are re-examined in light of our results and the implications for a coherent metabolic strategy (*sensu* Newell, 1980) are discussed. We hypothesize that a physiological surface such as the external respiratory surface or the absorptive area of the gut surface may limit growth and anabolism in *Capitella* species I.

## INTRODUCTION

Few studies have addressed the effects of temperature and body size on egestion rates within single species of deposit-feeding invertebrates (Hargrave, 1972; Hylleberg, 1975; Kudenov, 1982). This study was designed to measure changes in feeding rate *versus* body size at different temperatures for small, actively growing *Capitella* species I. This species has been shown to be one of a complex of sibling species, all formerly classified as *Capitella capitata*, each of which exhibits distinctive life history characteristics (Grassle and Grassle, 1976, 1977, 1978; Grassle, 1979). Throughout

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this paper we adopt the convention of referring to the worm by sibling species where it has been determined, and as *Capitella capitata* when referring to studies where the species is unknown.

Capitellid polychaetes typically occur, occasionally in great numbers, in organically enriched nearshore marine environments (*e.g.*, Grassle and Grassle, 1974; McCall, 1977; Pearson and Rosenberg, 1978; Rhoads *et al.*, 1978; Thistle, 1981). The genus *Capitella* has been termed an 'enrichment opportunist' by Pearson and Rosenberg (1978) and laboratory studies have documented that small individuals of *Capitella* sp. I can attain daily weight-specific growth rates as high as 21% (Tenore and Chesney, 1985). These characteristics make *Capitella* sp. I an ideal animal in which to study the allometry of feeding during the period of rapid growth and maturation.

Data on the allometry of feeding or egestion rate among species of deposit feeders indicates that feeding rate increases as a function of body size to a power less than one (Hargrave, 1972; Cammen, 1980). This means that larger animals will process relatively less sediment per unit weight than smaller animals. Hargrave (1972) discovered, in comparisons primarily among species of lacustrine and marine deposit feeders, that feeding rate scaled to approximately the 2/3 power of body weight. In a compilation of data encompassing 19 deposit-feeding species and spanning 3 phyla, Cammen (1980) found that organic matter ingestion was a function of body weight to the 0.74 power. These results suggest that egestion or feeding rate may be related to a physiological surface that scales to body size to approximately the 0.7 to 0.8 power (Pauly, 1981).

Studies of the allometry of egestion rate within a single species of polychaete, [the so-called 'dynamic' or ontogenetic allometry of Calder (1984)] indicate that the allometric exponent of feeding rate *versus* body size can be quite variable (Cadée, 1979; Hobson, 1967; Nichols, 1974; Kudenov, 1982; Dobbs and Scholly, 1986). These differences in the size-scaling of feeding rate may reflect differing metabolic strategies between species of infaunal polychaetes.

Several studies have measured the effect of temperature on feeding rate in polychaetes (Gordon, 1966; Cadée, 1976, 1979; Kudenov, 1982; Dobbs, 1983). Of these most were principally concerned with estimates of sediment reworking rate in the field, and the effect of temperature change was followed seasonally (Cadée, 1976, 1979; Kudenov, 1982). The effect of temperature may have been confounded with changes in food level and/or quality, worm density, and other random variables. Laboratory microcosm studies by Dobbs (1983) and Gordon (1966) have found that egestion rate varied linearly with temperature in *Clymenella torquata* and *Pectinaria gouldii*, respectively.

Polychaetes of the genus *Capitella* are often found in organic-rich environments which frequently have very low to nonexistent interstitial oxygen partial pressures (*e.g.*, Wells and Warren, 1975; Pearson and Rosenberg, 1978 and references therein). This suggests the possibility that *Capitella* species I may at times be limited energetically more by oxygen than by food supply in nature. Thus, an animal's ability to digest and absorb food would be greater than its ability to procure oxygen to efficiently metabolize food. One might also postulate an alternative hypothesis that growth and anabolism in *Capitella* sp. I are limited by food or energy availability (Tenore, 1975, 1977a, b, 1981; Tenore *et al.*, 1979; Tenore and Hanson, 1980). Under this hypothesis, at high food levels, anabolism would ultimately be limited by a surface area such as that of the gut absorptive area or the worm's ability to digest and absorb food or essential nutrients. The hypotheses of food or oxygen limitation need not be mutually exclusive, and indeed, interactions between the two may be important to animals in nature. A critical assumption of this type of analysis is that animals are built 'reasonably,' and that the evolutionary process will tend to minimize energy expenditure where possible. For example, if *Capitella* sp. I were energetically limited during growth by the allometry of gut or respiratory surface area, then the processing of additional sediment above an amount that could be effectively digested and absorbed, or metabolized by the oxygen reaching the mitochondria, would be an added cost with no additional benefit. One might then expect natural selection to reduce this added cost of feeding as body size increases through an egestion rate that scales in proportion to the relevant physiological surface(s).

We have performed experiments which indicate that egestion rate ($\mu g\ h^{-1}$) in *Capitella* sp. I scales as body size to the 0.70 power and increases exponentially as a function of acute temperature change. Data are also reported which directly measure the size-scaling of one potentially growth-limiting surface, that of external surface area, which scales as body size to the 0.77 power. These results are then compared to studies of respiration as a function of temperature in *Capitella* sp. I and other polychaetes. In addition, we discuss the results in view of the hypothesis that a physiological surface may limit growth or net energy gain in *Capitella* species I.

## MATERIALS AND METHODS

### *Sediments*

The sediment for all egestion rate experiments was obtained on 8 November 1983 from an intertidal mudflat located near the Flax Pond Marine Laboratory, Long Island, New York. The top 1–2 cm of mudflat sediment was sieved to <61 $\mu m$ on a Nitex screen, thoroughly homogenized, transferred to one pint freezer containers, and frozen at −20°C. Organic content of the sediment was estimated from weight loss on ignition (475°C, 6 h) after drying (60°C, 48 h) to be approximately 10%.

### *Laboratory culture conditions*

Small juvenile and adult *Capitella* species I individuals (obtained from Dr. K. Tenore) were used in all egestion rate experiments. The worms were maintained in mass culture in 720 $cm^2$ aerated plastic containers layered with 1–3 cm clean fine sand (<300 $\mu m$) at 15°C in a recirculating seawater aquarium. Animals were fed a 7 $g\ m^{-2}$ daily ration of Gerbers Mixed Cereal (Tenore, 1981). The cereal was first ground with a mortar and pestle and then stirred into the overlying culture water. Salinity in the mass culture varied between 25‰ and 35‰. The live worms used for the determination of the number of fecal pellets per gut and the number of fecal pellets per unit animal volume had been maintained in mass culture (28‰ S, 20°C) on Flax Pond silt-clay (<61 $\mu m$) surface sediment.

### *Determination of body-size relationships*

To determine the relation between body size and fecal pellet weight, between 200 and 800 fecal pellets (depending on size) were counted and filtered onto preweighed Nuclepore® membrane filters, rinsed with several ml 3% $NH_4COOH$ to remove salts, and dried for 24 hours at 55°C. The filters were then weighed on a Cahn 26 microbalance to ±1 $\mu g$ to measure pellet dry weight. Thus each point represents a value for several hundred individual pellets. To measure worm dry weight, preserved animals were placed on preweighed Nuclepore® filters, dried for 24 hours (55°C), and weighed to ±1 $\mu g$.

Animal volumes for egestion rate experiments (uncorrected for gut lumen) were measured by drawing the animals with the aid of a camera lucida and assuming a two-dimensional projection of a cylinder (Self and Jumars, 1978). To estimate the number of fecal pellets per unit body volume, live worms were removed from mass culture on silt-clay sediment (20°C) and rapidly transferred to glass petri dishes (4 cm diam.) where they were relaxed in isosmotic $MgCl_2$ solution. The number of fecal pellets in each worm gut is readily determined by flattening the worm under a glass cover slip. Fecal pellets defecated during the relaxation period were added to those counted within the gut. Body volumes of relaxed animals were determined in an analogous manner to those of preserved worms using a video camera mounted on a dissecting microscope. The precision of volume measurement for both preserved and relaxed worms is within 5%. In order to compare live and preserved worms we have assumed that fixation does not alter body volume.

Measurements of the projected area of the worms used in the feeding rate experiments were made by camera lucida drawings of the preserved worms.

### *Feeding rate* versus *temperature experiments*

The worms were removed from mass culture and transferred without food to the experimental temperature (15°, 20°, 25°C) for 24 hours prior to the start of each egestion rate experiment. Thus, the feeding rates measured at 20° and 25°C were based on acute temperature changes. Egestion rate experiments were run for either 6 (I, II, III, V) or 26 (IV) hours. The experimental salinities were 24‰ (III, IV) and 30‰ (I, II, V). Upon completion of each experiment animals were cleaned of adhering debris, fixed in a seawater solution of 20% buffered formaldehyde for 24 hours, and then transferred to 70% ETOH. All animals had a preserved body volume of between 0.27 and 2.62 $mm^3$. These body volumes correspond to worm lengths of approximately 1.0 to 15 mm. Feeding rate experiments (I through V) were conducted on worms placed singly in small (4 cm diameter) glassware dishes in 2–3 mm of Flax Pond silt-clay sediment. The animals were then allowed to feed undisturbed at the experimental temperature for 6 or 26 hours. None of the animals ever pelletized all of the available sediment. Feeding rate was determined by sieving (85 $\mu m$) and counting all fecal pellets defecated by an individual worm over the duration of the feeding experiment. Food density was manipulated by adding a known volume of non-nutritive diluent (clean glass beads) to sediment (1 beads:4 settled sediment, v/v). The glass beads added were in the range of particle diameters (3–85 $\mu m$) ingestible by *Capitella* sp. I of experimental size. Feeding rate was expressed as fecal pellets $animal^{-1} h^{-1}$.

### *Body size* versus *feeding rate*

A power function was fit to the data to describe the relationship between individual fecal pellet weight and worm volume. Plots of feeding rate ($\mu g$ sediment processed $h^{-1}$) and worm volume at the three experimental temperatures were made by multiplying the number of fecal pellets produced by an individual worm times the expected fecal pellet weight calculated from the relation between pellet weight and worm volume. Worm volume-specific [$\mu g$ dry weight (DW) sediment $(mm^3 \text{ worm})^{-1} h^{-1}$] egestion rate plots were made by dividing feeding rate ($\mu g\ h^{-1}$) by worm volume. Geometric mean (GM) regressions were used to construct all functional relationships while an ordinary predictive least-squares regression was used for the relation between preserved animal volume and dry weight (Ricker, 1973; 1984 and references therein).

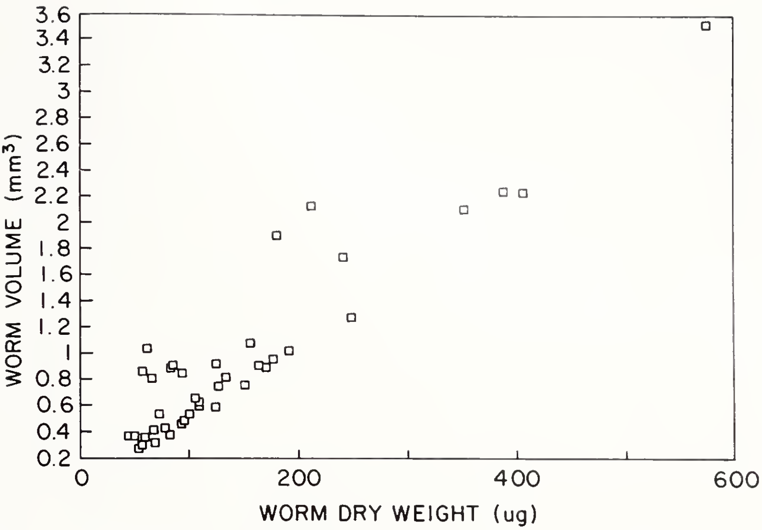

FIGURE 1. Relationship between estimated preserved volume and dry weight for *Capitella* species I. $V = 0.127 + 0.006DW$, $r^2 = 85.1\%$, $n = 40$; where V = worm volume ($mm^3$) and DW = worm dry weight ($\mu g$).

## RESULTS

### *Body-size relationships*

There is a positive linear relationship between individual worm dry weight and estimated body volume (Fig. 1). Individual fecal pellet weight was allometrically related to worm volume (Fig. 2). Large worms form relatively lighter fecal pellets. The relation between projected area and worm dry weight is shown in Figure 3. GM regression of the data indicates that projected area *Capitella* sp. I scales as dry weight to the 0.77 power. The number of pellets per unit live worm volume was found to decrease exponentially as body size increased (Fig. 4A). However, the relationship between the absolute number of pellets per worm and body volume was positive but nonsignificant ($r = .275$, $P > 0.05$, $n = 21$) (Fig. 4B). Thus, the number of pellets maintained on average within the guts of larger animals is at least equal to that of smaller animals. This is true in spite of the fact that the smallest worms measured had approximately ten times more pellets per unit body volume than the largest animals (Fig. 4A). The average number of pellets within the gut over the entire range of worm body sizes was 30.2.

### *Feeding rate* versus *temperature*

The results of egestion rate experiments I through V are given in Table I. Mean egestion rates ranged from approximately 13 pellets $animal^{-1}$ $h^{-1}$ at 15°C to 34 pellets $animal^{-1}$ $h^{-1}$ at 25°C. Egestion rate (pellets $animal^{-1}$ $h^{-1}$) did not vary with animal size ($r = -.109$, $P > 0.20$, $n = 42$). Differences in salinity or duration of the experiment had no influence on mean egestion rate (Table I). Experiments II and V were carried out under identical conditions with the exception that in experiment V the sediment was diluted with a known volume of glass beads. The worms showed no

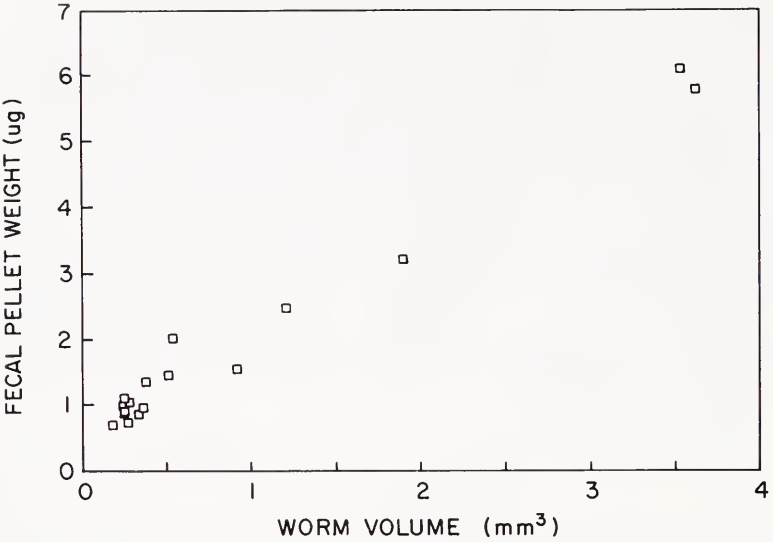

FIGURE 2. Allometric relation between individual fecal pellet weight and preserved *Capitella* sp. I volume. $PW = 2.270V^{0.701}$, $r^2 = 93.1\%$, $n = 17$; where PW = individual pellet weight ($\mu$g) and V = worm volume (mm$^3$).

difference in egestion rate between experiments II and V. Thus, *Capitella* sp. I did not alter its egestion rate in response to changes in food density. An exponential relationship exists between egestion rate and temperature (Fig. 5). The calculated $Q_{10}$ values for egestion rate between 15°C and 20°C and between 20°C and 25°C are 1.52

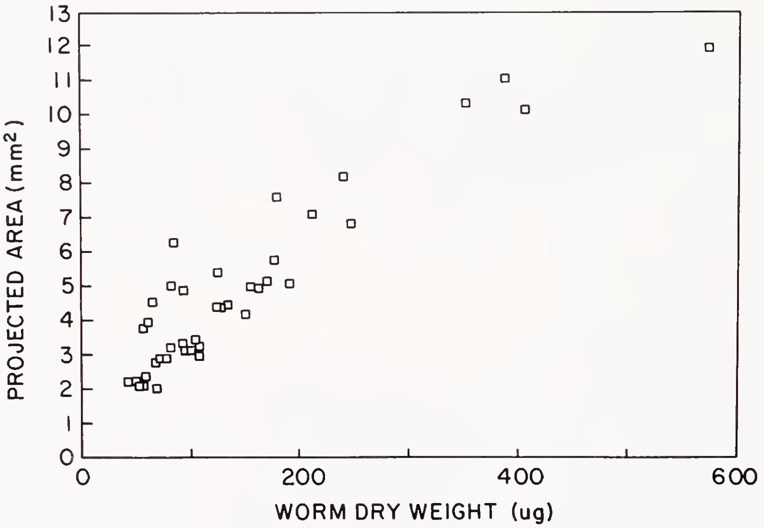

FIGURE 3. Allometric relation between worm projected area and dry weight. $A = 0.109DW^{0.772}$, $r^2 = 78.7\%$, $n = 40$; where A = projected area (mm$^2$) and DW = worm dry weight ($\mu$g).

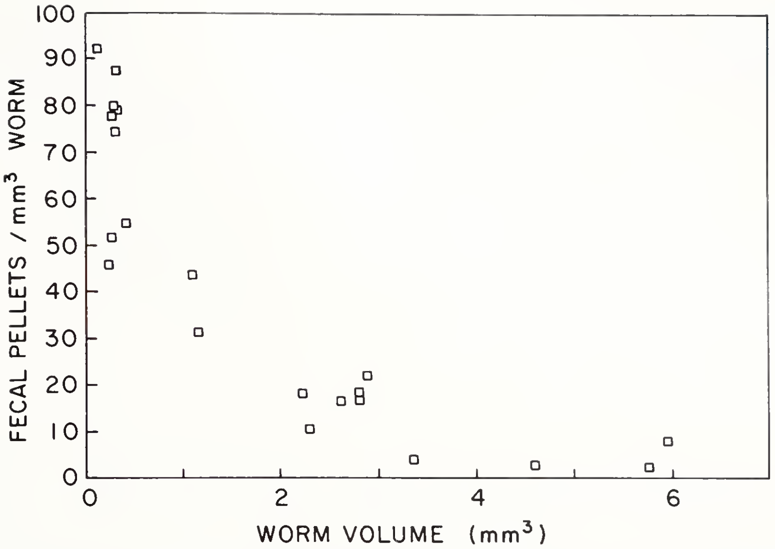

FIGURE 4a. Changes in the number of fecal pellets counted within the gut per unit worm volume of live *Capitella* species I. $F = 81.48\exp(-0.60V)$, $r^2 = 86.7\%$, $n = 21$; where $F = FP\ (mm^3\ worm)^{-1}$, $V$ = worm volume ($mm^3$).

and 4.07, respectively (Fig. 5). The $Q_{10}$ over the entire temperature range was 2.49. Thus, egestion rate ($\mu g\ h^{-1}$) could be predicted knowing only worm body size and temperature.

*Body size* versus *feeding rate*

Changes in egestion rate ($\mu g\ h^{-1}$) *versus* body size at the experimental temperatures are given in Figure 6. Estimates could be made in this way because pellet pro-

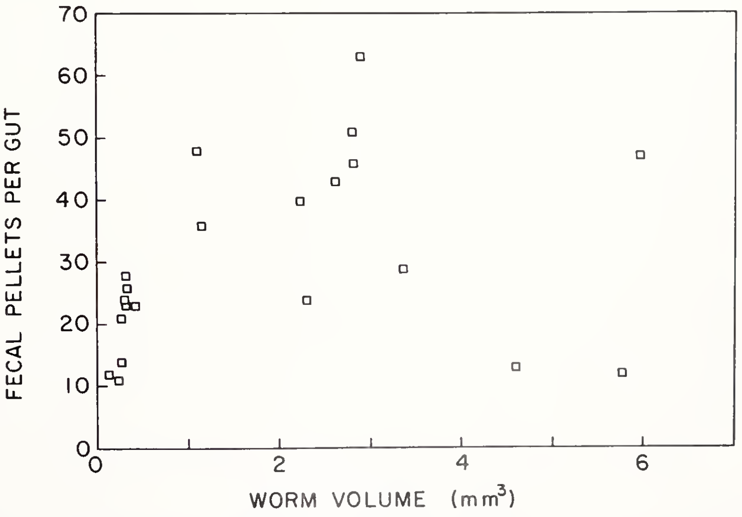

FIGURE 4b. Relationship between worm body volume and the number of fecal pellets counted within the gut ($r = 0.275$, $P > 0.05$, $n = 21$).

TABLE I

*Summary of egestion rate experiments*

| Expt. | Date | °C | S‰ | *Egestion rate* Mean (S.D.) (n) | Time (h) |
|---|---|---|---|---|---|
| I | 29 Nov 83 | 25 | 30 | 34.12 (12.99) (11) | 6 |
| II | 11 Feb 84 | 15 | 30 | 13.50 (7.657) (10) | 6 |
| III | 27 Sept 83* | 20 | 24 | 17.10 (5.167) (6) | 6 |
| IV | 27 Sept 83* | 20 | 24 | 16.70 (4.433) (6) | 26 |
|  | 27 Sept 83** | 20 | 24 | 16.90 (4.600) (12) | — |
| V | 19 Nov 83* | 15 | 30 | 13.94 (5.284) (10) | 6 |

Egestion rates are in fecal pellets per animal hour (mean) (S.D.) (n).
* Glass beads added.
** 27 Sept 83 experiments combined.

duction rate was independent of body size. The relationship between volume-specific egestion rate ([$\mu$g (mm$^3$ worm)$^{-1}$ h$^{-1}$]) and body size is shown in Figure 7. These data were fit to allometric power functions and the parameters are reported in Figures 6 and 7. Egestion rate is related to body size to the 0.70 power, indicating that large worms feed at a lower rate per unit body volume than small worms (Fig. 7). The fact that all curves for the three experimental temperatures have an identical slope when log-transformed indicates that a large animal has a greater absolute change in egestion rate with increasing temperature whereas a smaller worm has a greater relative change in egestion rate.

## DISCUSSION

### *Body size relationships*

Like many other deposit-feeding invertebrates, egestion rate in *Capitella* species I ($\mu$g DW sediment h$^{-1}$) is related to body size by an allometric exponent less than one

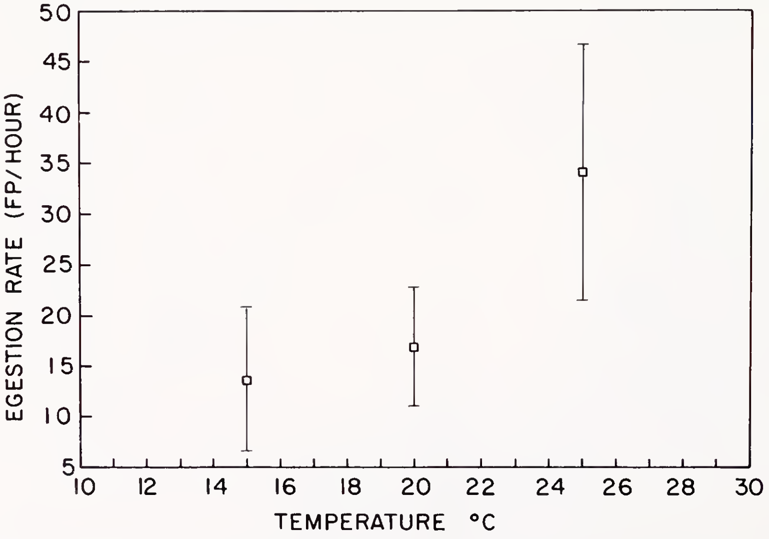

FIGURE 5. Changes in fecal pellet production rate with temperature. FP h$^{-1}$ = 0.94exp(0.15T), $r^2$ = 33.1%, n = 43; where T = °C. Error bars ± 1 S.D.

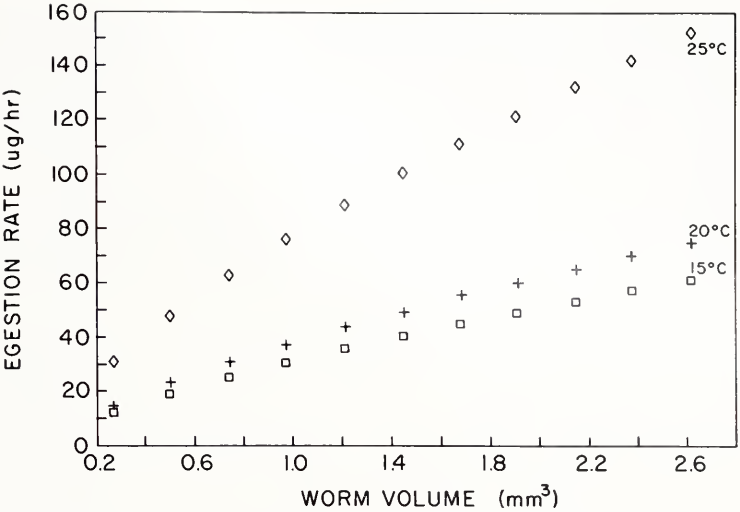

FIGURE 6. Calculated curves showing changes in estimated egestion rate *versus* worm volume at the three experimental temperatures. The parameters for the allometric functional relationships at the three experimental temperatures are a(15) = 31.14; a(20) = 38.36; a(25) = 77.45; b = 0.701. Where $\mu$g DW sediment $h^{-1}$ = $a(T)V^b$. V = worm volume (mm$^3$) and a(T) = temperature dependent constant.

(Hargrave, 1972; Cammen, 1980 and references therein). The change in the specific feeding rate of *Capitella* sp. I [$\mu$g DW sediment (mm$^3$ worm)$^{-1}$ $h^{-1}$] with body size is due entirely to two observed relationships: (1) Fecal pellet production rate does not

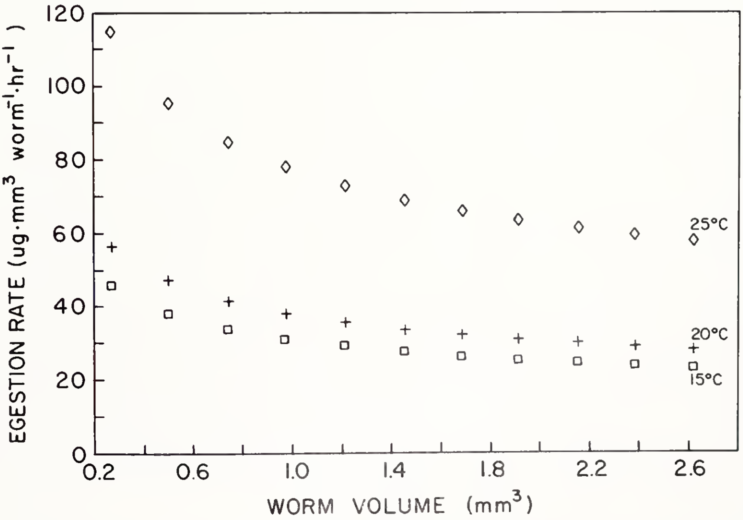

FIGURE 7. Calculated curves showing changes in estimated relative egestion rate with worm volume. The parameters for the allometric functional relationships at the three experimental temperatures are a′(15) = 31.14; a′(20) = 38.36; a′(25) = 77.45; b = −0.299. Where [$\mu$g dry weight sediment (mm$^3$ worm)$^{-1}$$h^{-1}$] = $a'(T)V^{b'}$. V = worm volume (mm$^3$) and a′(T) = temperature dependent constant.

change as a function of body size, and (2) smaller animals produce relatively heavier pellets (Fig. 2). This indicates that worms control egestion rate without changing pellet production rate, and therefore gut residence time, as they grow. Measurements made on individual *Capitella* species I during growth indicate that relative pellet weight is altered primarily by changes in pellet dimensions during ontogeny (Ms in prep.).

*Gut surface area and volume.* One potentially limiting physiological surface is that of the gut. It is possible that gut surface area limits the capacity of the worm to absorb food material. Nothing is presently known of how gut surface area might change with body size in *Capitella* sp. I. Direct measurements of gut surface area over a range of body sizes would be of great interest in this regard.

In *Capitella* species I, fecal pellets are formed within the first few setigers after ingestion of sediment (T. F. pers. obs.). Unless the digestion and absorption process occurs within the first few setigers, the worms must absorb food material from ingested sediment already in the form of compact fecal pellets. If there are physicochemical constraints (*e.g.,* rate of diffusion of enzymes etc.) pellet size (or weight) might reach an absolute limit in larger animals. However, recent data indicate that this is not the case and that the allometric relationship between pellet weight and worm body volume is maintained up to animal volumes of at least 10 $mm^3$ (Ms in prep.).

If the relative fullness (*i.e.,* fecal pellets/unit volume gut) of the gut of large worms is at least as great as that of small animals, then the exponential decline of relative pellet number with increasing body size (Fig. 4A) indicates that the percentage of the total worm volume occupied by the gut decreases as worms increase in size, meaning that gut volume will be related to total worm volume raised to a power less than one.

Additional data on pellet standing stocks within the gut of worms between the size of metamorphosis and approximately 1 $mm^3$ would be of great interest. Animals in this size range all have pellet standing stocks below the overall mean of 30 fecal pellets per gut. If this trend is real, and given the constant relationship between body size and pellet production rate, the average gut residence times of these smaller animals will be shorter than those of larger animals. These data further suggest that the growth of very small animals (less than 1 $mm^3$ body volume) may be limited by their ability to pack sediment into their gut and effectively absorb food. This is important because the time needed for digestion and absorption of food may impose additional constraints on the net energy intake of small worms, given their potentially shorter gut passage times. These small worms may be processing relatively greater amounts of sediment and retaining it for a shorter period within the gut. Larger animals do not show this trend, and growth in worms larger than approximately 1 $mm^3$ may be limited by other factors, such as oxygen uptake ability. In light of these considerations, absorption efficiency estimates for very small *Capitella* sp. I will be of great interest. Due to these potential constraints, recently metamorphosed worms may be particularly vulnerable to stresses imposed by food limitation or unfavorable temperatures. Metabolic stress may be further aggravated by the fact that oxygen solubility in seawater (35‰ S) decreases quite markedly with increasing temperature (approximately 30% from 10° to 30°C, DeJours, 1981). Small deposit feeders may not be scaled-down versions of adults. Juvenile animals may face fundamentally different problems and solve them in different ways.

*External surface area.* In order to obtain an estimate of external surface area in *Capitella* sp. I, we have assumed that external or respiratory exchange surface is proportional to worm projected area. In a study of the external surface of the aquatic oligochaete *Tubifex tubifex,* Kaster and Wolff (1982) found the posterior region of the worm (last 25 segments) to be about three times as surface-rich as the same length of worm at the anterior end. Unfortunately, no attempt was made to determine the size-scaling of this relationship. However, for the present discussion of the allometric arguments, and for the estimation of the scaling of external (respiratory) surface area in *Capitella* sp. I, it is not necessary to assume that the entire surface of the worm is used as an exchange surface, only that the proportion used as an exchange surface does not change during ontogeny. Given these assumptions, external surface area in *Capitella* sp. I will scale as body volume to the 0.77 power (Fig. 3). The similarity of the allometric exponents between the feeding rate and surface area functions indicates that feeding rate is proportional to, and indeed may be constrained by, external surface area. Two of the most likely ways by which external surface area might influence the scaling of egestion rate are through (a) limitation of transepidermal uptake of dissolved nutrients (DOM, vitamins) and/or (b) limitation of oxygen uptake for aerobic respiration.

(a) Uptake of dissolved nutrients. *Capitella capitata* has been shown to possess active uptake mechanisms for the transport of dissolved primary amines from interstitial water at a rate which approaches that calculated for metabolic oxygen consumption (Stephens, 1975). The uptake of dissolved nutrients probably does not amount to a large component of the nutritional strategy of *Capitella* sp. I because the worms shrink and lose weight exponentially when maintained in seawater without sediment (Cammen, 1985; Forbes and Lopez, unpub.). However, the possibility that some essential nutrient or 'vitamin' obtainable only through transepidermal uptake in dissolved form does limit growth or net energy gain, and thereby influences the scaling of feeding rate, cannot be ruled out.

(b) Uptake of dissolved oxygen. External surface area may limit oxygen uptake and therefore anabolism. A relationship of this type may have evolved under conditions of generally high food availability and chronically low oxygen tensions. Mangum (1976) states that "the greatest deprivation . . . that has accrued from the exploitation of the soft bottom was the loss of a microenvironment that remains constant in oxygen and virtually equilibrated to the atmosphere."

The cellular hemoglobin of *C. capitata* has a very high affinity for oxygen ($P_{50} = 3$ mm Hg; Wells and Warren, 1975) and sulfide has been shown to be a larval settlement cue for *Capitella* species I (Cuomo, 1985). These data indicate that the genus *Capitella* is adapted to exploit organic-rich low oxygen habitats. However, experiments by Warren (1977) and studies reviewed by Pearson and Rosenberg (1978) suggest that *Capitella capitata* is not exceptionally tolerant of low oxygen or high sulfide concentrations. Interstitial oxygen partial pressures from the organic sediments occupied by *Capitella capitata* are very low (8–12 mm Hg; Wells and Warren, 1975), suggesting that tube irrigation may be important in this species.

Wells (1949) was able to simultaneously measure irrigation and proboscideal activity in the polychaete *Arenicola marina* and found an inverse relationship with a periodicity of three to four minutes. That is, animals decreased feeding activity during irrigation episodes. A similar suspension of feeding activity occurs in *Capitella* species I during burrow irrigation (T.F. pers. obs.). These data suggest that cessation of feeding during periods of low ambient $O_2$ levels may be one mechanism by which decreased oxygen availability could act to suppress growth or anabolism.

*Coelomic space.* Another potential constraint affecting the feeding rate to body size relationship may be that animals must decrease the relative amount of space occupied by the gut within the coelom as they grow, and thereby decrease the relative pellet weight as they increase in size. This could be due to a demand for coelomic space not related to energy procurement or growth, such as the requirement for the production and development of eggs. Because sex determination in *Capitella* species I is probably a digametic system, with females as the heterogametic sex, all animals are either potential hermaphrodites or true females (Petraitis, 1985). Therefore, one would expect any constraints on total gut volume or width to operate on all animals, even functioning males, because they retain the potential to become hermaphrodites in the absence of females.

### *Feeding rate and temperature*

The calculated overall $Q_{10}$ value of 2.49 (Fig. 5) for the change in fecal pellet production rate with temperature from 15° to 25°C agrees well with values obtained for oxygen consumption in polychaetes (Coyer and Mangum, 1973) over similar temperature ranges. In general however, the values obtained for active and resting metabolism in polychaetes are quite variable. For example, Coyer and Mangum (1973) found $Q_{10}$ values ranging from 1.81 to 4.56 over 12.5° to 27.5°C for the increase in resting metabolism of *Glycera dibranchiata.*

In a recent study, Cammen (1985) estimated metabolism in *Capitella* species I by a variety of methods (oxygen uptake, weight loss, metabolic carbon loss), over a wide range of body sizes. GM regressions were calculated for experiments performed at 10°, 20°, and 30°C. Allometric coefficients and exponents calculated for each regression were also given (Cammen, 1985; Table I). It is thus possible to calculate, using data from the $O_2$ uptake method, metabolic carbon loss for a 200 $\mu$g individual between 15° and 25°C. This calculated loss will be directly proportional to oxygen uptake. The $Q_{10}$ value calculated from Cammen's data from 15° to 25°C is 2.82. The agreement with the change of feeding rate with temperature calculated in this study ($Q_{10} = 2.49$) is thus quite close. This is somewhat surprising given the fact that in one study the temperature change was acute (this study) and in the other more gradual acclimation occurred (Cammen, 1985). Increases in metabolic rate with temperature seem to be closely paralleled by concomitant increases in feeding rate. It does not appear [based on calculations made from data in Cammen (1985), for a 200 $\mu$g DW worm] that *Capitella* sp. I is able to show a compensatory response in metabolic rate with increases in temperature. That is, metabolic carbon loss increases exponentially with temperature, even in worms acclimated to experimental temperatures for 11 to 16 days. One can also calculate from Cammen's (1985) $O_2$ uptake data that temperature has a greater effect on the metabolic carbon loss (*i.e.,* metabolism) of smaller worms. The rate-temperature (RT) curves of metabolic carbon loss increase exponentially with increasing temperature for an animal of given size. More interesting, however, is the fact that the rate of increase (*i.e.,* the exponential coefficient of temperature, K) is greater for smaller animals. We have plotted the calculated values of K *versus* body size over a wide range of body sizes in Figure 8. *Capitella* sp. I may thus face an energetic 'bottleneck' during ontogeny due to the fact that the effect of unfavorable temperature or food conditions may have a much greater impact on the small animals. However, under conditions of abundant food, the high metabolic rate of small animals may result in the very high growth rates observed in the laboratory (21% per day for small worms; Tenore and Chesney, 1985).

The inability to adjust metabolic energy expenditure with increasing temperature, particularly evident in small worms, is indicative of an 'exploitative' metabolic strategy (Newell, 1979, 1980). Briefly, species adopting an exploitative strategy are adapted to an abundant food supply, and exhibit high rates of energy turnover and production. Energy conservation brought about by metabolic compensation under conditions of high food availability may not be the most efficient strategy for animals

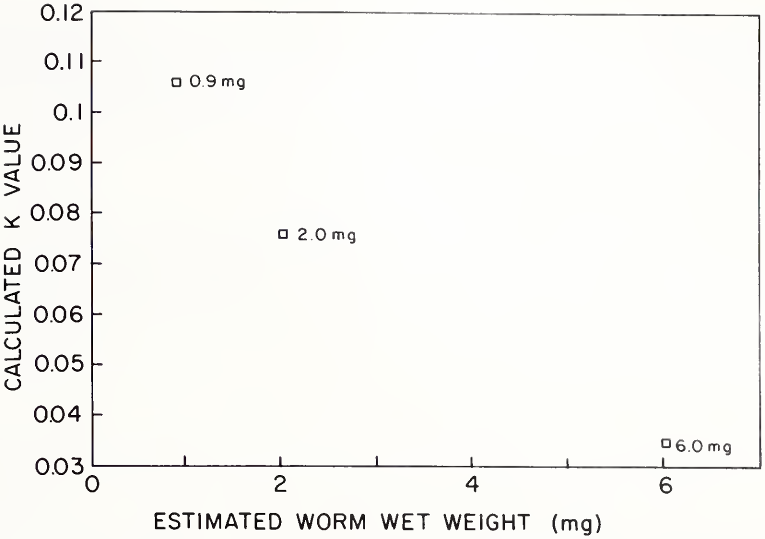

FIGURE 8. Calculated changes in the exponential coefficient K with body size. Where MR = C [exp(KT)]; MR = $\mu$g carbon released $day^{-1}$ $worm^{-1}$, T = temperature °C. C = constant. Data from Cammen, 1985.

tending to grow rapidly and reproduce quickly (Newell, 1979). Animals adapted to exploiting food-rich habitats may be unable to make adjustments in metabolic systems that are designed to make the most out of environmentally favorable situations. Homeostatic changes in such metabolically important parameters as the activities of key enzyme systems, or the number of mitochondria per cell, in response to fluctuating environmental conditions may not be possible for some of the so-called opportunistic or 'weedy' species. Shumway *et al.* (1983) have found that the opportunistic bivalve *Mulinia lateralis* maintains elevated activity even under conditions of anoxia. The clams maintain a high level of metabolism and do not seem capable of adjustment in the face of low oxygen levels. *M. lateralis* appears to be adapted to high levels of food availability, and when food level declines, characteristic mass mortalities may occur (Shumway and Newell, 1984). It may be that species such as *Capitella* sp. I and *Mulinia lateralis,* by adopting physiologies that maximize energy turnover rates at high food levels, are then incapable of adjustment in response to a decrease in food or oxygen supply.

## *Summary*

Egestion rate ($\mu$g $h^{-1}$) in *Capitella* species I scales as body size to a power less than one. This is due entirely to two observed relationships: (1) Fecal pellet production rate does not change as a function of body size, and (2) smaller worms produce relatively heavier pellets. We discuss several hypotheses that may explain why such relationships have evolved. For example, egestion rate may be functionally related to a limiting physiological surface such as: (1) Gut surface area, or (2) external respiratory surface area. Relative pellet size ([$\mu$g $(mm^3 worm)^{-1}$]) also may be limited by the need for coelomic space for the development of the ovary.

Changes in measured egestion rates (fecal pellets $h^{-1}$) closely parallel those of met-

abolic carbon loss over similar temperatures. However, there is a greater rate of increase with temperature in smaller animals (data from Cammen, 1985). This suggests the possibility of an energetic 'bottleneck' during ontogeny. Smaller animals may be affected to a greater degree (both positively and negatively) by the prevailing food and temperature regime.

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Reference: *Biol. Bull.* **172:** 202–211. (April, 1987)

# THE PREDOMINANCE OF CLONES IN POPULATIONS OF THE SEA ANEMONE *ANTHOPLEURA ASIATICA* (UCHIDA)

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## ABSTRACT

To assess the population structure of the sea anemone *Anthopleura asiatica,* sex ratios and acrorhagial responses were examined among ten populations in the Seto Inland Sea of Japan. In spite of the fact that all specimens were capable of eliciting an acrorhagial response on contact with other individuals of the same species, no response was found in certain populations. These populations were always composed of individuals of the same sex. This result indicates that examination of the response was effective in distinguishing clones of the anemone. On the basis of sex and acrorhagial response, each of the populations seems to consist of a single or a few clones, and moreover, one clone appears to be distributed over 5 km through six separate locations. Diversity of clones among locations may imply sexual recruitment in the past. The anemones probably prosper asexually in local populations while dispersal to new habitats is facilitated by sexual reproduction. The causes of the clones' predominance and the applicability of the Strawberry-Coral Model to the anemone are discussed.

## INTRODUCTION

Some sea anemones can reproduce in a variety of ways both sexually and asexually (Chia, 1976). It is interesting to study how these anemones use their sexual and asexual reproductive modes in their life cycles, because such studies may provide useful information which helps to answer the questions about the roles of sex (Williams, 1975). Genotypic structure of a population is a consequence of reproductive modes. However, few studies have examined sea anemone population structure: *Anthopleura elegantissima* (Francis, 1973a; Sebens, 1982), *Haliplanella luciae* (Shick, 1976; Shick and Lamb, 1977), *Metridium senile* (Hoffmann, 1976; Shick *et al.,* 1979; Bucklin and Hedgecock, 1982) and *Actinia tenebrosa* (Black and Johnson, 1979; Ayre, 1983, 1984).

Aggressive behavior using acrorhagi borne under tentacles has been known in sea anemones for some time (Abel, 1954; Bonnin, 1964; Francis, 1973b; Bigger, 1976, 1980; Brace and Pavey, 1978; Ottaway, 1978; Williams, 1978; Ayre, 1982). Acrorhagial responses are elicited by contact with other anemones of the same species and with other cnidarian species. On the other hand, it is known that no response occurs among clonemates, which have the same genotype. Therefore, the response between anemones must be useful to recognize clones and to assess the structure of populations (Sebens, 1982), although the usefulness of this distinguishing method may be limited (Bigger, 1980; Ayre, 1982).

*Anthopleura asiatica,* a common intertidal sea anemone in western Japan, repro-

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Contribution no. 278 from the Mukaishima Marine Biological Station.

duces asexually by longitudinal fission, as well as sexually (Uchida, 1958, 1965). On the rocky shore of Matsugahana Pt. near Mukaishima Marine Biological Station, Japan, the anemones live in tidepools with their pedal discs in contact with each other (Fujii, 1985). When I examined anemone gonads, in 1983, to assess the reproductive cycle of anemones inhabiting the rocky shore, the noticeably mature specimens were always males (Fujii, in prep.).

The present study examines the sex ratio and acrorhagial responses of *A. asiatica* specimens at ten locations in the central Seto Inland Sea of Japan (Table I, Fig. 1), in order to assess the population structure of the anemone. The effectiveness of the acrorhagial response in distinguishing clones, the population structure, and the applicability of the Strawberry-Coral Model (Williams, 1975) to the species, are discussed on the basis of these results.

## MATERIALS AND METHODS

Between August and September 1983, collections of *Anthopleura asiatica* individuals were made from ten locations which differed in size and habitat type: Kamiebujima Islet, Matsugahana Pt., Kannonzaki Pt., Sasajima Islet, Shijushima Islet, Iwashijima Is., Ategijima Islet, Toyoshima Is., Takaikamishima Is., and Aoshima Is. (Fig. 1, Table I). Although I also visited other locations on these and other islands, *A. asiatica* was only found at these ten locations. At three of them, Kannonzaki Pt., Toyoshima Is., and Aoshima Is., the anemones were found only in one or a few tidepools, while at the others, they were distributed over more than ten meters of shoreline. The anemones often form aggregations of contiguous individuals. In each location, the specimens were collected randomly from several aggregations which had been chosen throughout the habitats. The specimens from each aggregation were transported separately to the laboratory in labeled plastic bags and kept in seawater in labeled plastic cups (8 cm in basal diameter, 5 cm in depth) until they were examined. The seawater was changed daily.

Acrorhagial responses were examined using the specimens of the three aggregations which had been furthest away from each other in each location. Each anemone was allowed to attach to a piece of oyster shell in the cup, so that it could be moved easily. After two days, firmly attached anemones were paired with the specimens from the other two aggregations at the same location. A 1-second touch technique (Bigger, 1980) was applied to test the response. The pair's tentacles were lightly touched to each other for one second every fifteen seconds. Successive contacts were made ten times between the same tentacle tips, or until an acrorhagial response occurred. Over three repetitions were done in every three combinations. If no response was elicited in a pair, the pair was placed with their tentacles in contact and observed for more than one hour.

The acrorhagial response also was examined between anemones from different locations, using specimens from the three aggregations. Twenty-nine combinations were tested with at least five repetitions of each, using different pairs (Fig. 3).

Collected anemones, including those used for the response test, were all anaesthetized in the cups with a 50:50 solution of seawater and 7.5% (w/v) magnesium chloride. Well-relaxed anemones were cut longitudinally and their gonads were examined under a stereomicroscope.

In November 1983, another collection of anemones was made from five of the ten locations, Kamiebujima Islet, Matsugahana Pt., Sasajima Islet, Shijushima Islet, and Ategijima Islet, in order to examine the acrorhagial responses again. Specimens were collected from 6, 20, 13, 5, and 9 aggregations, respectively (five specimens at

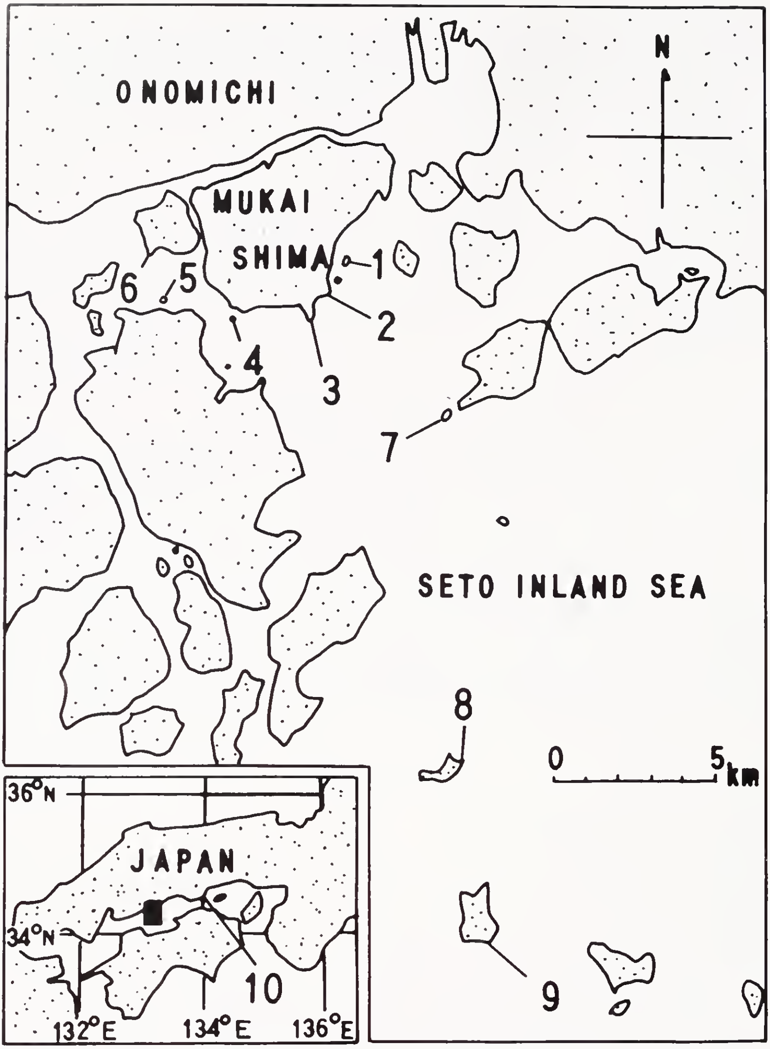
ONOMICHI
MUKAI SHIMA
N
1
2
3
4
5
6
7
8
9
SETO INLAND SEA
0 5 km
36°N
34°N
JAPAN
10
132°E
134°E
136°E

FIGURE 1. The map of the ten locations.

each aggregation). The responses were examined with every possible combination of the aggregations in each location and those of the location pairing their representatives. If no response occurred between their representatives, the rest of the specimens from different locations were set in contact with each other in a vessel and observed to confirm the results.

## RESULTS

The sex of 408 specimens of *A. asiatica* was determined from 62 aggregations and they were found to be dioecious: 76 females, 281 males, 51 immature (Table II). Ripe individuals were easily sexed by the color and form of the gonads. Females' gonads

TABLE I

*Description of locations*

| Locations | Ranges (area or length) | Habitat types |
|---|---|---|
| (1) Kamiebujima Islet | 20 m | t = p = r |
| (2) Matsugahana Pt. | 2000 m$^2$ | t ≫ r > p |
| (3) Kannonzaki Pt. | 0.35 m$^2$ | t |
| (4) Sasajima Islet | 50 m | r ≫ p |
| (5) Shijushima Islet | 12 m | p > t |
| (6) Iwashijima Is. | * 22 m | t |
| (7) Ategijima Islet | 40 m | p ≫ r = t |
| (8) Toyoshima Is. | * 9 m$^2$ | t |
| (9) Takaikamishima Is. | * 250 m | p ≫ r = t |
| (10) Aoshima Is. | 6 m$^2$ | t |

Relative abundance of inhabited habitants are represented by inequality signs. t: tidepool, p: pebble, r: rock. *After this study, the anemones were found at the next shores outside the reported ranges.

were filled with light blue eggs, and the white vesicles in the males were filled with sperm.

The sex ratios in most locations were extremely skewd (Table II). Only a single sex was found in eight of the ten locations. All specimens from six locations, Kamiebujima Islet, Mastugahana Is., Kannonzaki Pt., Sasajima Islet, Shijushims Islet, and Ategijima Islet, were males, while all specimens from two locations, Toyoshima Is. and Aoshima Is., were females. Although both sexes were found in the other two locations, Iwashijima Is. and Takaikamishima Is., even there, the specimens from an aggregation were always of one sex (Table II).

No acrorhagial responses were elicited in some combinations of the specimens; their tentacles were interlaced for over one hour without any response. But all anemones used in this study apparently had the potential to display the acrorhagial response because they always elicited the responses after several artificial tentacle contacts (from one to four times) in the other combinations. The process of the response in *A. asiatica* was similar to those in the other acrorhagi-bearing anemones (Bonnin, 1964; Francis, 1973b; Bigger, 1980): (1) tentacle withdrawing after contact; (2) expansion of acrorhagi; (3) leaning to the opponent; (4) application of acrorhagial ectoderm; and (5) recovery.

In each of the eight locations where all specimens were the same sex, no acrorhagial response was found in any pair of the three aggregations (Fig. 2). Additional examinations of acrorhagial response were carried out in five of the eight locations, combining more aggregations, but no response occurred in any of them (Table III). In fact, the responses were always found in two of the three combinations in the two locations where there were both males and females (Fig. 2), but even there, the specimens of the two aggregations which did not respond to each other were always the same sex.

As for acrorhagial responses between anemones from different locations, twenty-nine combinations were examined (Fig. 3). In this test the responses between males (Ategijima Islet *vs.* other male locations) and those between females (Aoshima Is. *vs.* Iwashijima Is.) were also observed. The acrorhagial response between anemones seems to be independent of their sex. The responses were elicited in every combination of the locations except those of the locations along Mukaishima Is., where all

TABLE II

*Distributions of sexes in the ten locations*

| | | Aggregations | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Locations | | a | b | c | d | e | f | g | h | i | j | k | l | m | n | o | Total | |
| (1) Kamiebujima | M | 5 | 5 | 3 | 5 | 5 | 10 | 5 | 5 | | | | | | | | M | 43 |
| | F | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | | | | | | F | 0 |
| | I | 0 | 0 | 2 | 0 | 0 | 0 | 2 | 0 | | | | | | | | I | 4 |
| (2) Matsugahana | M | 6 | 5 | 4 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 4 | 5 | 25 | 9 | M | 98 |
| | F | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | F | 0 |
| | I | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 8 | 27 | I | 37 |
| (3) Kannonzaki | M | 4 | | | | | | | | | | | | | | | M | 4 |
| | F | 0 | | | | | | | | | | | | | | | F | 0 |
| | I | 2 | | | | | | | | | | | | | | | I | 2 |
| (4) Sasajima | M | 5 | 5 | 5 | 5 | 5 | | | | | | | | | | | M | 25 |
| | F | 0 | 0 | 0 | 0 | 0 | | | | | | | | | | | F | 0 |
| | I | 0 | 0 | 0 | 0 | 0 | | | | | | | | | | | I | 0 |
| (5) Shijushima | M | 7 | 7 | 7 | 7 | 7 | 9 | | | | | | | | | | M | 44 |
| | F | 0 | 0 | 0 | 0 | 0 | 0 | | | | | | | | | | F | 0 |
| | I | 0 | 0 | 0 | 0 | 2 | 1 | | | | | | | | | | I | 3 |
| (6) Iwashijima | M | 0 | 4 | 5 | 3 | | | | | | | | | | | | M | 12 |
| | F | 8 | 0 | 0 | 0 | | | | | | | | | | | | F | 8 |
| | I | 0 | 0 | 0 | 0 | | | | | | | | | | | | I | 0 |
| (7) Ategijima | M | 8 | 11 | 6 | 5 | 5 | | | | | | | | | | | M | 30 |
| | F | 0 | 0 | 0 | 0 | 0 | | | | | | | | | | | F | 0 |
| | I | 0 | 0 | 0 | 0 | 0 | | | | | | | | | | | I | 0 |
| (8) Toyoshima | M | 0 | 0 | 0 | 0 | 0 | | | | | | | | | | | M | 0 |
| | F | 4 | 5 | 5 | 5 | 2 | | | | | | | | | | | F | 21 |
| | I | 1 | 0 | 0 | 0 | 3 | | | | | | | | | | | I | 4 |
| (9) Takaikamishima | M | 5 | 5 | 5 | 0 | 0 | 0 | 0 | 5 | 5 | | | | | | | M | 25 |
| | F | 0 | 0 | 0 | 5 | 5 | 5 | 5 | 0 | 0 | | | | | | | F | 20 |
| | I | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | | | | | I | 0 |
| (10) Aoshima | M | 0 | 0 | 0 | 0 | | | | | | | | | | | | M | 0 |
| | F | 5 | 5 | 5 | 12 | | | | | | | | | | | | F | 27 |
| | I | 0 | 0 | 1 | 0 | | | | | | | | | | | | I | 1 |

M: male, F: female, I: immature.

specimens were males. In order to make sure of the results, particularly the exception, additional examinations were done combining five locations; four of them along Mukaishima Is. No response was also found among the four locations while the responses were always elicited in combinations between the fifth location, Ategijima Islet, and one of the other four (Table III).

## DISCUSSION

Sea anemones bearing acrorhagi display aggressive behavior on contact with the same species or with other cnidarian species (Bigger, 1980). This study shows that *A. asiatica* responds aggressively to other individuals of the species with the same pattern of behavior as other acrorhagus-bearing anemones (Abel, 1954; Bonnin, 1964; Francis, 1973b; Bigger, 1976, 1980; Ottaway, 1978; Williams, 1978; Ayre, 1982).

Acrorhagial response seems to be useful to the assessment of the genetical struc-

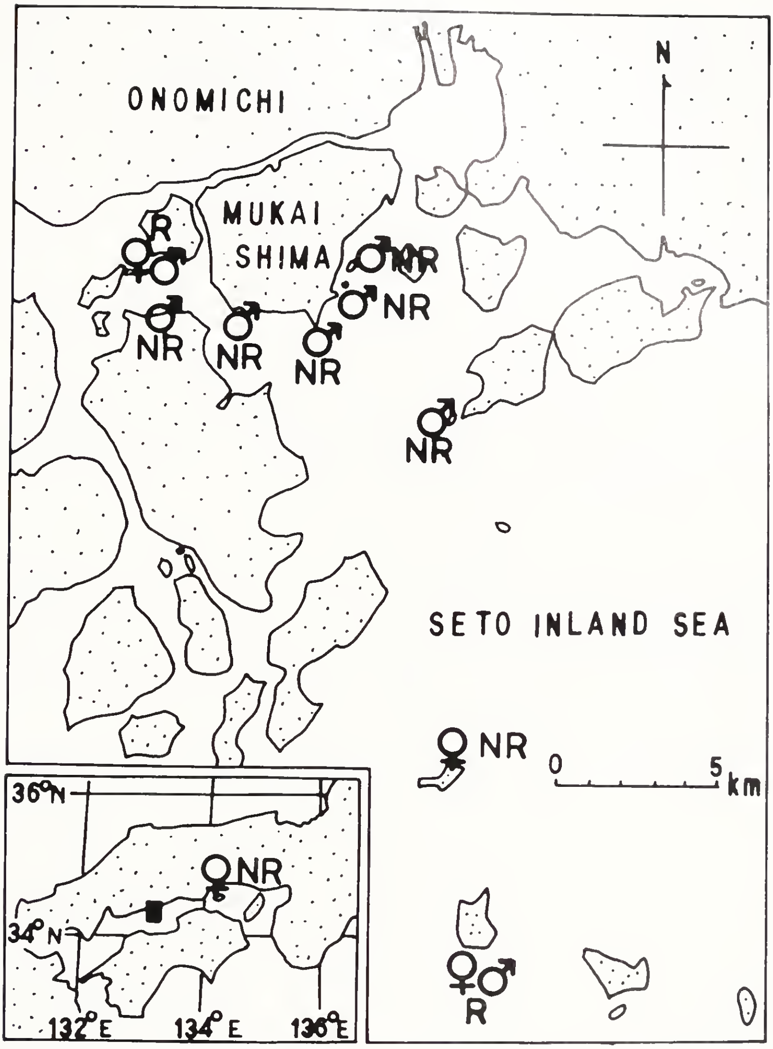

FIGURE 2. Acrorhagial responses among three aggregations in each location. R: the responses occurred in two of three combinations of aggregations. NR: no response was elicited. Signs of sexes represent the sex compositions of the locations.

ture of anemone populations because anemones are able to distinguish between clonemates and non-clonemates (Francis, 1973a, b, 1976; Bigger, 1976, 1980; Ayre, 1982), although their distinguishing ability may be limited. Ayre (1982) failed to produce aggressive responses on contact between some genetically dissimilar individuals of *Actinia tenebrosa*. When we use the acrorhagial response to distinguish clones, it is important to know to what extent we can trust the response.

A clone of dioecious animals is composed of a single sex. Therefore distribution of the sexes can be a useful indication of asexual reproduction, especially in sessile animals such as anemones (Ford, 1964; Sebens, 1982). The exceedingly skewed sex ratios in the locations (Table II) suggest that the anemones reproduce asexually there.

TABLE III

*Results of the second acrorhagial response test within and between five locations*

| | (1) | (2) | (4) | (5) | (7) |
|---|---|---|---|---|---|
| (1) Kamiebujima Is. | –<br>C(6.2) | – | – | – | + |
| (2) Matsugahana Pt. | | –<br>C(18.2) | – | – | + |
| (4) Sasajima Islet | | | –<br>C(13.2) | – | + |
| (5) Shijushima Islet | | | | –<br>C(5.2) | + |
| (7) Ategijima Islet | | | | | –<br>C(9.2) |

–: no responses were elicited in the combination, +: the responses were always elicited. The number of tested combinations within locations were represented by C(N.2) = (N!/(N − 2)!)/2, where N is the number of collected aggregations. As for the test between locations, at least five repetitions were done. All specimens of (1)–(5) were mingled in a vessel without any response.

The acrorhagial responses of anemones are thought to be independent of their sexes (Ayre, 1982; Sebens, 1984). In this study, *A. asiatica* responded aggressively to both male and female specimens from different clones. Therefore, assuming the test of the response was ineffective in distinguishing clones, one would expect to find individuals of both sexes among the anemones that did not respond to each other. However, anemones that did not respond were always of the same sex (Fig. 2, 3). This indicates that *A. asiatica*'s ability to distinguish clones is very good.

Asexual reproduction has a great influence on genetical structure of a population. Sea anemones which reproduce asexually have less genotypic diversity in a population than would be expected in a sexual population with free recombination and random matings (Shick and Lamb, 1977; Black and Johnson, 1979; Ayre, 1983; Hoffmann, 1986; Shick and Dowse, 1985). The results of this study indicate that the populations of *A. asiatica* have extremely low diversities. Eight of the ten locations seem to consist of single clones, although some minor clones may have been missed. Even the other two locations seem to consist of a few dominant clones. Moreover, this study suggests that one of the clones may dominate at six locations, covering a range of over five kilometers (Fig. 3). This might be explained by a tidal current passing through the locations, but more evidence will be needed to substantiate this idea. It has been reported that the sea anemones, *A. elegantissima* and *Metridium senile,* form clonal aggregations (Ford, 1964; Francis, 1973; Hoffmann, 1976; Sebens, 1982) and a clone of them can be distributed over a relatively large area in their populations (Shick *et al.,* 1979; Sebens, 1982). It has also been shown that the populations of the sea anemone *H. luciae,* introduced on the Atlantic coast of North America, are composed of one or a few clones (Shick, 1976; Shick and Lamb, 1977; Shick *et al.,* 1979). However, the formation of discrete colonies by a single clone had not been reported until now.

As Shick *et al.* (1979) suggested for *H. luciae,* single clone predominances in *A. asiatica* may be caused by one or more of the following: (1) the clone best adapted to the habitats was selected; (2) there were few immigrants and new settlements so that the few lucky anemones arriving on unoccupied habitats could prosper and predominate there, or (3) each clone at a location had excluded others aggressively. Ayre (1985) revealed through a field transplantation experiment that populations of *A.*

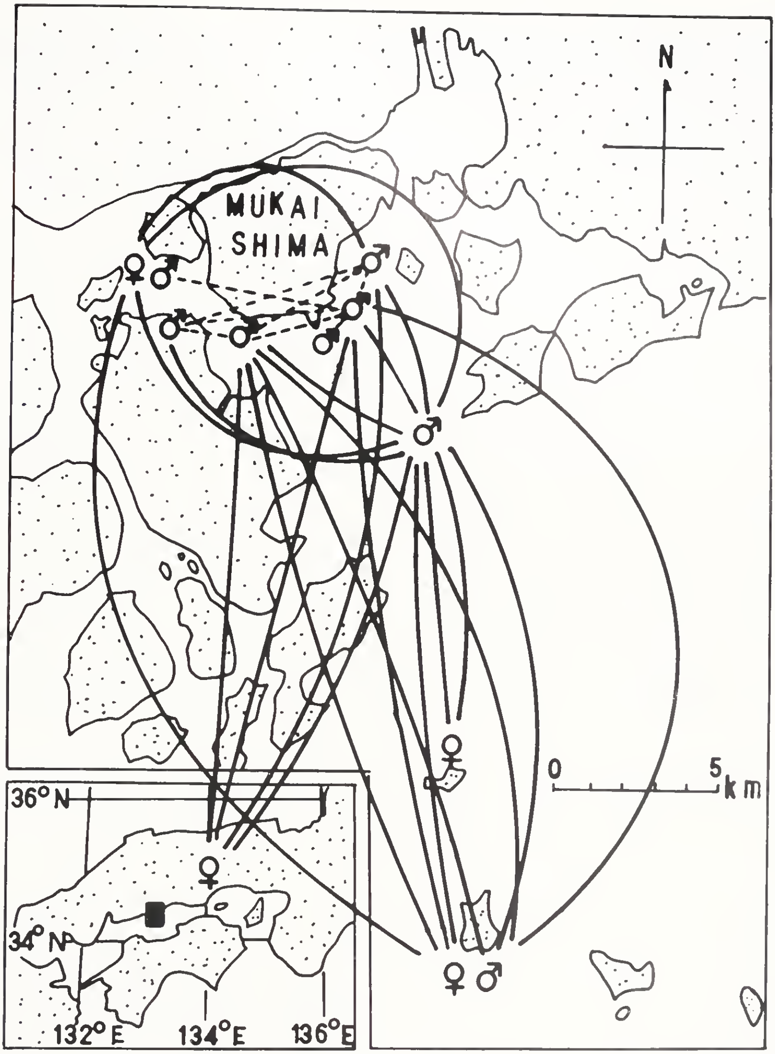

FIGURE 3. Acrorhagial responses among locations. No response occurred between the locations connected by broken lines. The responses occurred between the locations connected by solid lines.

*tenebrosa* consist of highly locally adapted clones. The anemones showed higher asexual fecundity when they were transplanted into their original habitats. In the center of the Inland Sea, populations of *Anthopleura asiatica* seem to consist of one or a few clones. Even if the clones are the best adapted to their locations, they probably do not have narrow but rather wide adaptational ranges, because each location consists of a variety of habitat types (*e.g.,* tidepool, rock, and boulder at different tidal heights). The clones of the colonizing anemone, *H. luciae,* are adapted to a wide range of temperature and salinity (Shick, 1976; Shick and Lamb, 1977; Shick and Dowse, 1985). Shick *et al.* (1979) revealed that in *H. luciae,* the preadaptations of some clones to low temperature allowed their predominance. Although such a severe condition could not be found out in the Seto Inland Sea, there may be a different kind of limiting condition which functions as a bottleneck. A variety of clones would be expected in an unstable habitat (Sebens, 1982; Ayre, 1984). Because the predominance of a single or a few clones occurs even on the boulder beaches, which seem to be unstable, it also seems plausible that the predominant clones started as a few lucky founders which encountered unoccupied habitats. A low rate of new recruitment may allow the clones to prosper in diverse and unstable habitats. Aggressive interactions of anemone are thought to play an important role in intra-specific competition for space (Francis, 1973a, b Ayre, 1983). But it is still uncertain whether one clone can exclude others completely from its habitats through aggressive interactions. In fact, Bigger (1980) suggested that acrorhagial response can be viewed as one of a set of ecological facters which may maintain a genetic heterogeneity. In any case, they may serve to repel the other invading clones once a clone occupies a habitat (Sebens, 1982).

The present study suggests not only asexual proliferation of *A. asiatica* in the local populations but also their sexual settlement in the past. On the basis of the aggressive response, each location seems to be occupied by different clones, with the exception of the locations around Mukaishima Is. (Fig. 3). This diversity was probably brought about through sexual reproduction. *A. asiatica* seems to use sexual reproduction for wide dispersal while using asexual reproduction in local proliferation.

The life-history pattern of *A. asiatica* appears to fit the predictions of the Strawberry-Coral Model which Williams (1975) proposed to overcome the theoretical disadvantage of sex. The model assumes that clonal proliferation of sessile organisms is limited by their habitat space, and that there is an intense selection, so that only the progeny with sufficiently high local fitness can win in each habitat. In the model, diverse sexual progeny are predicted to have a greater probability of establishing new colonies outside the limits of original habitats, because of the different demands of new habitats.

In spite of the conformity in the life history pattern, some situations of *A. asiatica* may be inconsistent with the assumptions of the model. For the start, a clone of the species seems to inhabit a variety of habitat types. From this situation, it is likely that the clones were not the fittest but just the lucky ones (intense competition may be rare) and/or that each clone has a wide adaptational range (*i.e.,* variability may be less important). Thus the model may not be applicable in each case. Aggressiveness may be another problem (Ayre, 1983), because it does not seem to be dependent on environmental conditions. If the most aggressive clone was selected in every habitat, the introduction of variation through sexual reproduction might be disadvantageous.

## ACKNOWLEDGMENTS

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# APPLICATION OF AN IMPROVED METHOD OF TWO-DIMENSIONAL ELECTROPHORESIS TO THE SYSTEMATIC STUDY OF HORSESHOE CRABS

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## ABSTRACT

Protein constituents of skeletal and cardiac muscles of extant horseshoe crabs, *Tachypleus tridentatus, T. gigas, Carcinoscorpius rotundicauda,* and *Limulus polyphemus,* were examined by two-dimensional gel electrophoresis in order to investigate the applicability of this technique in systematics and to obtain more data on phylogenetic relationships of the four species. Electrophoretic patterns were analyzed by both comparison of spot positions only and that of presumed protein groups (positions and shapes).

Our phenetic analyses confirmed the disparate position of the North American species, *L. polyphemus,* and the similarity of the three Asian species. Our cladistic analysis suggested a sister-group relationship of *T. tridentatus* and *C. rotundicauda.* This suggestion, however, was based on a small number of characters.

On the basis of this electrophoretic study, we suggest that conventional native (non-denaturing) gel electrophoresis for enzymes is optimal for measuring genetic differences among organisms at lower taxonomic levels, but two-dimensional gel electrophoresis is best for overall protein comparison.

## INTRODUCTION

There are four currently recognized extant species of horseshoe crabs, *Tachypleus tridentatus, T. gigas, Carcinoscorpius rotundicauda,* and *Limulus polyphemus. T. hoeveni,* described by Pocock (1902), was considered by Waterman (1958) to be a synonym of *T. gigas.*

Previous phylogenetic studies on those four species have utilized external morphology, hybridization tests, and amino acid sequencing (Pocock, 1902; Sekiguchi and Sugita, 1980; Shishikura *et al.,* 1982; Srimal *et al.,* 1985). These studies have shown that *Limulus polyphemus,* which inhabits the east coast of North America, is disparate from the three southeast Asian species.

The classical or morphological view places two of these Asian species in the genus *Tachypleus* and considers *Carcinoscorpius* to be a monotypic genus (Pocock, 1902; Størmer, 1952). Contradictory biochemical, embryological, and genetical data, however, suggest that *T. tridentatus* is more similar to *C. rotundicauda* than to *T. gigas* (Sekiguchi and Sugita, 1980; Shishikura *et al.,* 1982; Srimal *et al.,* 1985).

Since previous biochemical studies on horseshoe crabs have dealt only with two proteins, coagulogen and hemocyanin (Shishikura and Sekiguchi, 1978; Sugita and

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Sekiguchi, 1981; Shishikura *et al.*, 1982; Srimal *et al.*, 1985), the number of protein characters examined has been too small for reasonably deducing phylogenetic relationships. Because two-dimensional gel electrophoresis can detect more subtle protein differences than conventional electrophoresis, it can provide information on many kinds of proteins. However, the usefulness of the data for phylogenetic analyses has not yet been examined (Wiley, 1981).

In this study, proteins from cardiac and skeletal muscles of the four species of horseshoe crabs were separated by two-dimensional gel electrophoresis (1) to examine its applicability to phylogenetic analysis, (2) to obtain new data on phylogenetic relationships among the three Asian species, and (3) to re-examine the disparate taxonomic position of the North American species. We also compared the applicability of the two-dimensional gel electrophoresis method with that of the conventional method which utilizes a native (non-denaturing) gel. Previous studies have suggested that the two-dimensional method gives a smaller magnitude of both intraspecific and interspecific differences than does the latter method (Brown and Langley, 1979; McConkey *et al.*, 1979; Walton *et al.*, 1979; Racine and Langley, 1980; Aquadro and Avise, 1981; McConkey, 1982).

## MATERIALS AND METHODS

*Tachypleus tridentatus* was collected in Imari, Japan. *Tachypleus gigas* and *Carcinoscorpius rotundicauda* were obtained from Bangsaen, Thailand. *Limulus polyphemus* was purchased from the Marine Biological Laboratory, Woods Hole, Massachusetts, USA.

The tubular heart (cardiac muscle) and a pair of skeletal muscles that connect the prosoma with gills were dissected from each specimen and cleaned quickly in filtered seawater to prevent contamination from other tissues and hemolymph. The heart was cut into anterior, central, and posterior parts for comparison of protein components in respective sections. The heart parts and skeletal muscles were cut into small pieces and stored at −80°C.

Two-dimensional electrophoresis was carried out essentially according to the method improved by Hirabayashi (1981). One piece (0.1 g) of each muscle was homogenized with a Dounce homogenizer in 4 ml of an extraction medium of 8 $M$ urea, 1 $M$ KI, 0.1 m$M$ $N^{\alpha}$-tosyl-L-lysylchloromethanehydrochloride, 10% $\beta$-mercaptoethanol, and 0.1 $M$ Tris-HCl, pH 7.5. This homogenate was dialyzed against 7 $M$ urea for 3–4 hours and centrifuged at 30,000 × *g* for 30 minutes with a Beckman JA-14 rotor. For single species runs, 60 $\mu$l and 40 $\mu$l of the supernatants from cardiac and skeletal muscles, respectively, were loaded on the first dimension isoelectric focusing gels. When samples from two species were compared, a 1:1 mixture of 80 $\mu$l (40 $\mu$l from each heart) or 50 $\mu$l (25 $\mu$l from each skeletal muscle) was also loaded. Isoelectric focusing was carried out at 500 V for 24 hours with cardiac muscle and 30 hours with skeletal muscle. The remaining supernatants and precipitates were each mixed with a hot 1% agarose solution containing 2% SDS, 10% glycerol, 5% $\beta$-mercaptoethanol, 0.02% bromophenol blue, and 0.05 $M$ Tris-HCl, pH 6.8. Each mixture was chilled in a glass tube and stored as reference for total supernatant or precipitate components of the second dimension SDS-polyacrylamide gel electrophoresis. The second dimension, with a concentration gradient of acrylamide (12–20%), was carried out at 30 mA, when the bromophenol blue tracking dye was in the stacking gel, and then continued at 60 mA until the dye reached the lower end of the running gel.

For optimal comparison of two-dimensional patterns, we electrophoresed extracts from each of two species and a mixture of the two at the same time ("triplet method," Fig. 1). A close examination of the mixture pattern (Fig. 1b) was indispensable to confirm overlapping (or sharing) of spots suspected to be similar from individual patterns (Fig. 1a, c). Preliminary examination of cardiac muscle with the "triplet method" did not show significant differences among the three male and one female specimens of *T. tridentatus* and among the anterior, central, and posterior parts of a single heart of the animal. However, we used only the anterior part, since the sample was large enough. For interspecific analyses, we used six "triplet" combinations to compare patterns among the four species. In all, about 100 obvious spots were examined on each pattern of cardiac and skeletal muscles for each of the four species. We only used those spots that could be consistently matched or clearly defined.

The two-dimensional electrophoresis method, applied herein, has two advantages over those used in previous studies. First, the substitution of agarose for acrylamide in the first dimension gel allowed us to analyze proteins of higher molecular weights (up to about 250 KD). Second, we used four kinds of ampholytes (rather than one or two) in the first dimension for wider pH coverage (pH 3.5–9.5). These changes, plus a concentration gradient of acrylamide in the second dimension, permitted us to analyze about 100 proteins, presenting many more characters for systematic study than other methods of electrophoresis.

## RESULTS

Typical two-dimensional electrophoresis patterns of cardiac muscle of the four species are shown in Figures 1 and 2. Ninety-seven of about 100 major spots for each of the four species were "shared" (that is, having overlapping positions) by at least two species. These spots were numbered in the order of their relative molecular weights (Fig. 3). Those shown in boxes below Nos. 5 and 6 (Fig. 2) may have been derived from hemolymph proteins, probably hemocyanin. Hemolymph could not be removed completely, even though the muscle was thoroughly washed in seawater.

The sharing (overlapping) of cardiac muscle spots by the four species is shown in Table I, where the numbers correspond to those in Figure 3. The numbers of spots shared by any two of the three Asian species were almost the same and roughly twice those shared by each Asian species and the North American one (Table II).

When only the spot position was considered, we regarded two similar-appearing spots separately even if both were presumed to be derived from the same kind of proteins by their similar morphology and positions relative to other spots. Nos. 20 and 21, for example, were counted separately for analysis of the position only (Table I), although they may have been counterparts.

Next we considered such spots (*e.g.,* Nos. 20 and 21) as belonging to the same protein group and counted all examined spots as representing protein groups (Table III). In some instances, no spot was shared for the presumed protein group, *e.g.,* Nos. 98–108 in Table III. This resulted in a difference of five shared spots for cardiac muscle between the two approaches (see Tables I and III).

Tabulation of shared cardiac muscle protein spots (Table IV) gave a similar result to that when only the spot position was considered (Table II), the usual method of analyzing two-dimensional electrophoretic patterns. Therefore, both approaches support the view that the three Asian species are more similar to one another than to the North American species.

Cardiac muscle was used in this study both because of its importance to life and the homogeneity of its cell population. For similar reasons, we also examined skeletal

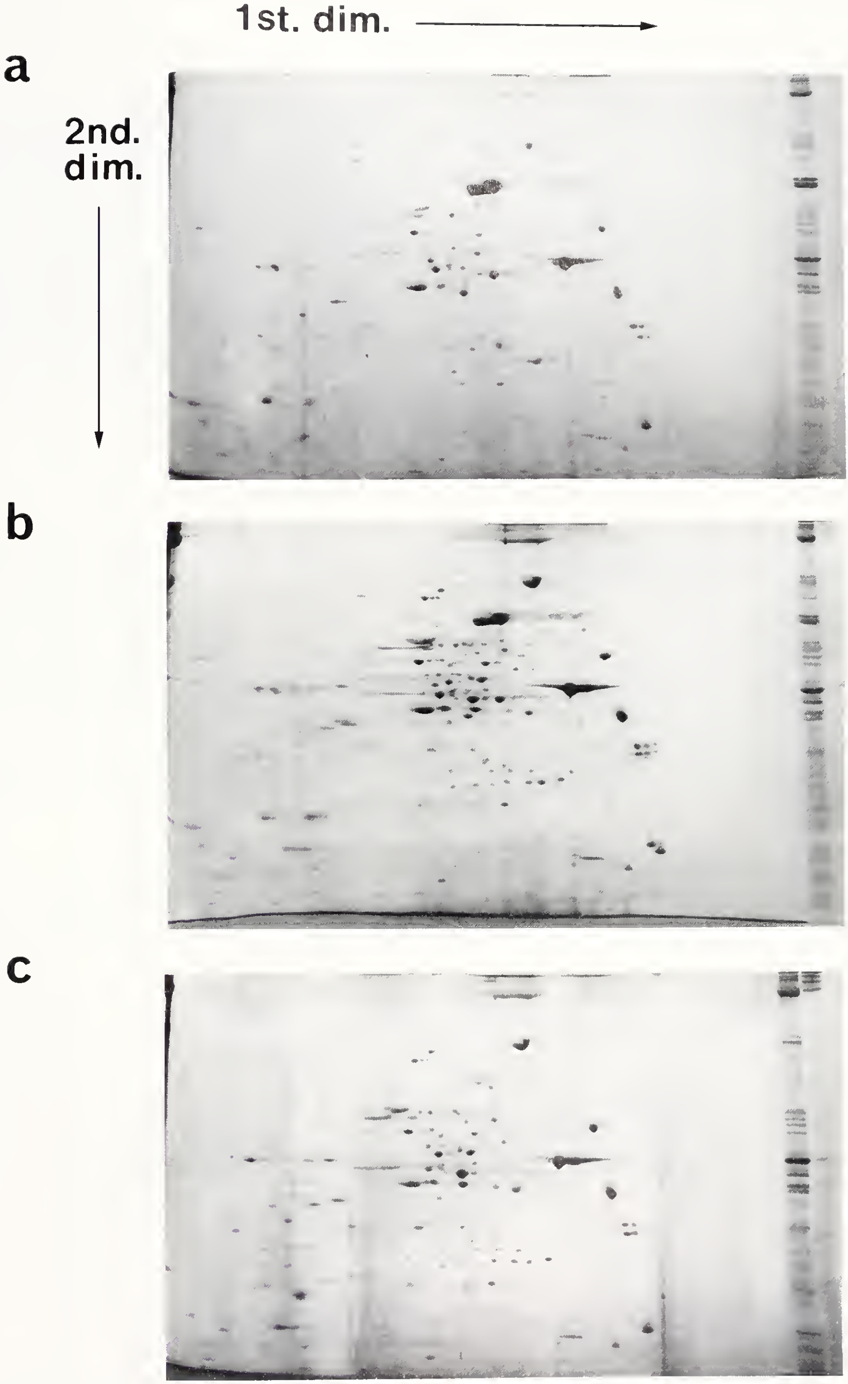


FIGURE 1. The "triplet patterns" of cardiac muscle. For optimal comparison of two-dimensional patterns, we electrophoresed extracts from each species (a, c) and a mixture of the two (b). a, *Tachypleus tridentatus;* c, *Limulus polyphemus.* Two lanes at the right-hand end of each pattern are references for total supernatant (left) and precipitate (right) components.

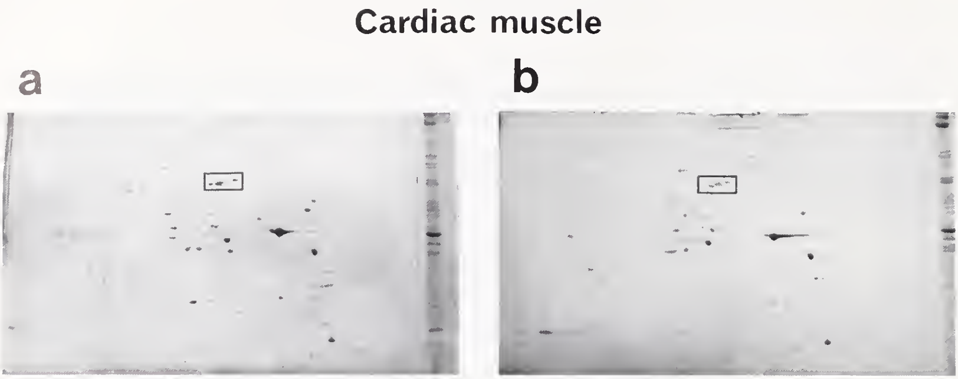
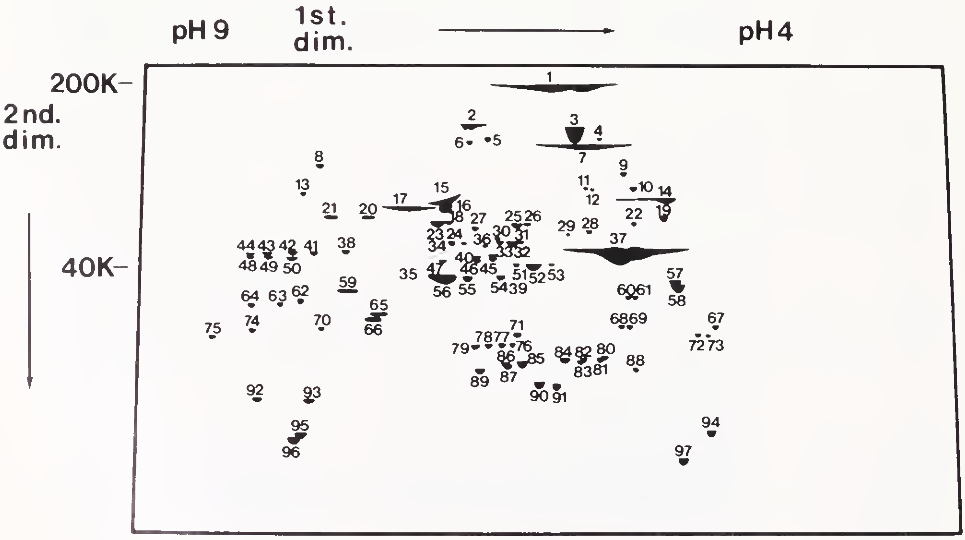

Cardiac muscle
a
b

FIGURE 2. The typical two-dimensional electrophoresis patterns of cardiac muscle. a, *Tachypleus gigas;* b, *Carcinoscorpius rotundicauda.* The spots in boxes were probably derived from hemolymph proteins.

muscle that connects the prosoma with the gills. With skeletal muscle, 97 of about 100 major spots on two-dimensional electrophoresis patterns (Fig. 4) were recognized as shared (*i.e.*, having overlapping positions) between at least two species, and shared spots by each pair of species were counted as before (see also Table II). Re-examination for shared spots in protein groups (*i.e.*, similar spot morphology and positions) gave a similar result (Table IV), although it differed by 22 spots. Skeletal muscle patterns also confirm the greater similarity among the Asian species when compared to the North American species.

Using the results in Table IV, we quantified the similarity among the four species


pH 9
1st. dim.
pH4
200K
2nd. dim.
40K
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2
3
4
6
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FIGURE 3. The schematic drawing of 97 spots of cardiac muscle. These spots, numbered in the order of their relative molecular weights, were shared by at least two of the four species.

TABLE I

*Sharing of spots (positions) for cardiac muscle*

| Species | No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *T. tridentatus* |  | + | + | + |  | + | + | + | + | + | + | + | + | + | + | + |  | + | + |  | + |  | + | + | + | + |
| *T. gigas* |  | + |  | + | + | + | + | + | + | + | + |  |  | + | + |  | + | + | + | + |  | + | + | + | + | + |
| *C. rotundicauda* |  | + | + | + | + | + | + | + |  | + | + | + | + |  |  | + | + | + | + | + | + |  |  | + | + |  |
| *L. polyphemus* |  | + |  | + |  | + |  | + |  | + | + |  |  |  |  |  |  | + | + |  |  | + |  | + | + | + |

| Species | No. | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 | 43 | 44 | 45 | 46 | 47 | 48 | 49 | 50 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *T. tridentatus* |  | + | + |  |  | + | + | + | + | + | + |  | + |  | + | + | + | + | + |  | + | + |  | + | + | + |
| *T. gigas* |  |  | + | + | + |  | + | + |  | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |  |
| *C. rotundicauda* |  |  | + | + | + |  | + | + |  | + | + | + | + | + | + |  | + | + | + |  | + | + | + | + | + | + |
| *L. polyphemus* |  | + | + |  |  | + |  | + | + |  |  | + | + | + |  | + |  | + | + | + |  |  |  |  |  |  |

| Species | No. | 51 | 52 | 53 | 54 | 55 | 56 | 57 | 58 | 59 | 60 | 61 | 62 | 63 | 64 | 65 | 66 | 67 | 68 | 69 | 70 | 71 | 72 | 73 | 74 | 75 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *T. tridentatus* |  | + | + | + | + | + | + | + | + | + | + | + | + |  | + | + | + | + |  | + | + | + |  |  | + | + |
| *T. gigas* |  | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + |  | + | + | + | + | + | + | + | + |
| *C. rotundicauda* |  | + |  |  | + |  | + | + | + | + | + | + | + | + | + | + | + |  | + | + | + | + | + | + | + |  |
| *L. polyphemus* |  |  |  |  | + |  | + | + | + |  |  |  |  |  |  |  | + | + |  |  |  | + |  |  |  |  |

| Species | No. | 76 | 77 | 78 | 79 | 80 | 81 | 82 | 83 | 84 | 85 | 86 | 87 | 88 | 89 | 90 | 91 | 92 | 93 | 94 | 95 | 96 | 97 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *T. tridentatus* |  | + | + | + | + |  | + | + | + | + | + | + |  | + | + | + |  | + | + | + | + | + | + |
| *T. gigas* |  | + | + | + | + | + |  | + |  | + |  |  | + | + |  |  | + | + | + | + |  | + |  |
| *C. rotundicauda* |  | + | + | + |  |  | + | + | + | + | + | + |  | + | + |  | + | + | + | + | + |  |  |
| *L. polyphemus* |  |  |  |  |  | + | + |  | + | + | + | + | + |  |  | + |  |  | + |  | + |  | + |

The symbol (+) represents the presence of a spot shared by at least two of the four species on the electrophoresis pattern of the species.

TABLE II

*Number of shared spots*

| Combination | Cardiac muscle | Skeletal muscle |
|---|---|---|
| *T. tridentatus* and *T. gigas* | 62 | 71 |
| *T. tridentatus* and *C. rotundicauda* | 62 | 68 |
| *T. gigas* and *C. rotundicauda* | 63 | 65 |
| *T. tridentatus* and *L. polyphemus* | 36 | 46 |
| *T. gigas* and *L. polyphemus* | 31 | 47 |
| *C. rotundicauda* and *L. polyphemus* | 30 | 45 |

by applying the formula: $F = 2N_{xy}/(N_x + N_y)$ after Aquadro and Avise (1981). In this formula, F is the similarity between species x and y, $N_{xy}$ is the number of spots shared by x and y, and $N_x$ and $N_y$ are the numbers of spots scored for x and y, respectively. These calculations, shown in Table V, revealed that the similarity between any two of the three Asian species approximated 0.6 for both skeletal and cardiac muscles. Between the North American species and each Asian species, the similarity for cardiac and skeletal muscles was about 0.35 and 0.45, respectively.

If electrophoretic data, when analyzed phenetically, refutes the classical phylogenetic hypothesis, the data should be carefully examined for possible false similarities due to retention of ancestral character states (Baverstock *et al.*, 1979). Both Farris (1974) and Baverstock *et al.* (1979) showed that cladistic analysis of electrophoretic data can give phylogenetic relationships that differ from those inferred from the data analyzed phenetically.

In general, electrophoretic data is analyzed phenetically using the similarity-clustering method. However, phylogenetic relationships, when based only on the similarity, make the unverifiable assumption that evolutionary rates of organisms are uniform. If evolutionary rates are not uniform, two differentiated species, which have evolved at relatively slow rates, may show many shared characters (*i.e.*, spots on electrophoresis patterns) due to retention of ancestral character states. At the same time, one of two closely related species, which has evolved relatively fast, may show, as derived character states, many unshared characters (spots). This may lead to the false conclusion that the two closely related species (of the three) are remote and the two differentiated species are similar. It is therefore important to subject electrophoretic data to cladistic analysis without assuming that evolutionary rates are uniform. However, to identify the ancestral or derived character state for each group of proteins is difficult. As pointed out by Baverstock *et al.* (1979), only the outgroup criterion is suitable for recognizing the character states with electrophoretic data.

Accepting their opinion and using the principle that derived character states held in common imply phylogenetic relationships, we analyzed our electrophoretic data for both cardiac (Table III) and skeletal muscles cladistically by regarding *Limulus polyphemus* as the outgroup, since no previous study has contradicted its disparate position relative to the three other species. The results of our cladistic analysis showed a sister-group relationship between *Tachypleus tridentatus* and *Carcinoscorpius rotundicauda* among the three Asian species. However, this conclusion is based on 1 of the 42 protein groups examined for cardiac muscle (Nos. 20–21 in Table III) and on 3 of 51 for skeletal muscle, since these were the only ones where we could regard overlapping spots as derived character states held in common.

Comparison of spot positions only is less complicated and more suitable for phe-

Table III

*Sharing of spots in presumed protein groups for cardiac muscle*

<table>
<tr><th>Species</th><th>No.</th><th>1</th><th>2</th><th>3</th><th>4</th><th>5</th><th>6</th><th>7</th><th>8</th><th>9</th><th>10</th><th>11</th><th>12</th><th>13</th><th>14</th><th>15</th><th>16</th><th>17</th><th>18</th><th>19</th><th>20</th><th>21</th><th>22</th><th>23</th><th>24</th><th>25</th><th>26</th><th>27</th></tr>
<tr><td>T. tridentatus</td><td></td><td>+</td><td>+</td><td>+</td><td>○</td><td>+</td><td>+</td><td>+</td><td>+</td><td>+</td><td>+</td><td>+</td><td>+</td><td>+</td><td>+</td><td>+</td><td>○</td><td>+</td><td>+</td><td>○</td><td colspan="2">+</td><td>+</td><td>+</td><td>+</td><td>+</td><td>+</td><td>+</td></tr>
<tr><td>T. gigas</td><td></td><td>+</td><td>○</td><td>+</td><td>+</td><td>+</td><td>+</td><td>+</td><td>+</td><td>+</td><td>+</td><td></td><td>○</td><td>+</td><td>+</td><td>○</td><td>+</td><td>+</td><td>+</td><td>+</td><td colspan="2">*</td><td>+</td><td>+</td><td>+</td><td>○</td><td>○</td><td>+</td></tr>
<tr><td>C. rotundicauda</td><td></td><td>+</td><td>+</td><td>+</td><td>+</td><td>+</td><td>+</td><td>+</td><td></td><td>+</td><td>+</td><td>+</td><td>+</td><td></td><td>○</td><td>+</td><td>+</td><td>+</td><td>+</td><td>+</td><td colspan="2">+</td><td>○</td><td>+</td><td>+</td><td>△</td><td>△</td><td>+</td></tr>
<tr><td>L. polyphemus</td><td></td><td>+</td><td>△</td><td>+</td><td>△</td><td>+</td><td>○</td><td>+</td><td></td><td>+</td><td>+</td><td></td><td></td><td></td><td>△</td><td>△</td><td>△</td><td>+</td><td>+</td><td>△</td><td colspan="2">*</td><td></td><td>+</td><td>+</td><td>+</td><td>+</td><td>+</td></tr>
</table>

| Species | No. | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 | 39 | 40 | 41 | 42 | 43 | 44 | 45 | 46 | 47 | 48 | 49 | 50 | 51 | 52 | 53 | 54 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *T. tridentatus* | | ○ | | + | + | + | + | + | + | ○ | + | | + | + | + | + | + | | + | + | | + | + | + | + | + | + | + |
| *T. gigas* | | + | + | ○ | + | + | ○ | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | + | | + | + | + | + |
| *C. rotundicauda* | | + | + | △ | + | + | △ | + | + | + | + | + | + | | + | + | + | | + | + | + | + | + | + | ○ | ○ | ○ | + |
| *L. polyphemus* | | △ | | + | ○ | + | + | ○ | ○ | + | + | + | ○ | + | ○ | + | + | + | | ○ | | | ○ | | △ | △ | △ | + |

| Species | No. | 55 | 56 | 57 | 58 | 59 | 60 | 61 | 62 | 63 | 64 | 65 | 66 | 67 | 68 | 69 | 70 | 71 | 72 | 73 | 74 | 75 | 76 | 77 | 78 | 79 | 80 | 81 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *T. tridentatus* | | + | + | + | + | + | + | + | + | | + | + | + | + | ○ | ○ | + | + | ○ | ○ | + | + | + | + | + | + | | + |
| *T. gigas* | | + | + | + | + | + | + | + | + | + | + | + | + | ○ | + | + | + | + | + | + | + | + | + | + | + | + | + | |
| *C. rotundicauda* | | ○ | + | + | + | + | + | + | + | + | + | + | + | △ | + | + | + | + | + | + | + | ○ | + | + | + | ○ | | + |
| *L. polyphemus* | | △ | + | + | + | ○ | ○ | | ○ | ○ | | | + | + | △ | △ | | + | △ | △ | ○ | | ○ | ○ | ○ | △ | + | + |

| Species | No. | 82 | 83 | 84 | 85 | 86 | 87 | 88 | 89 | 90 | 91 | 92 | 93 | 94 | 95 | 96 | 97 | 98 | 99 | 100 | 101 | 102 | 103 | 104 | 105 | 106 | 107 | 108 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *T. tridentatus* | | + | + | + | + | + | | + | + | + | ○ | + | + | + | + | + | + | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ | ○ |
| *T. gigas* | | + | | + | ○ | ○ | + | + | ○ | | + | + | + | + | | + | ○ | △ | △ | △ | △ | △ | △ | △ | △ | △ | | △ |
| *C. rotundicauda* | | + | + | + | + | + | | + | + | | + | + | + | + | + | | △ | ▽ | ▽ | ▽ | ▽ | ▽ | ▽ | ▽ | | ▽ | △ | ▽ |
| *L. polyphemus* | | | + | + | + | + | + | ○ | △ | + | △ | | + | ○ | + | | + | □ | □ | □ | | □ | □ | □ | ▽ | □ | ▽ | □ |

The symbols (+) and (*) represent the presence of a spot shared by at least two species on the pattern of the species, that is, they show the presence of a protein electrophoretically common to that of other species in a group of proteins with probably identical character. The symbols (○), (△), (▽), and (□) show the presence of an unshared spot on the pattern of the species. A blank indicates the absence of a spot.

TABLE IV

*Number of shared spots in presumed protein groups*

| Combination | Cardiac muscle | Skeletal muscle |
|---|---|---|
| *T. tridentatus* and *T. gigas* | 60 | 62 |
| *T. tridentatus* and *C. rotundicauda* | 60 | 57 |
| *T. gigas* and *C. rotundicauda* | 62 | 60 |
| *T. tridentatus* and *L. polyphemus* | 36 | 41 |
| *T. gigas* and *L. polyphemus* | 30 | 43 |
| *C. rotundicauda* and *L. polyphemus* | 30 | 39 |

netic analysis than use of protein groups (positions and shapes). However, the latter can be used for both cladistic and phenetic analyses.

## DISCUSSION

Our phenetic analyses support the view that *Limulus polyphemus* is disparate from the three Asian species, as previously reported (Pocock, 1902; Shuster, 1962; Shishikura and Sekiguchi, 1978; Sekiguchi and Sugita, 1980; Dorai, *et al.,* 1981; Sugita and Sekiguchi, 1981; Shishikura *et al.,* 1982; Yamamichi and Sekiguchi, 1982; Srimal *et al.,* 1985). These analyses also show that the three Asian species are equally

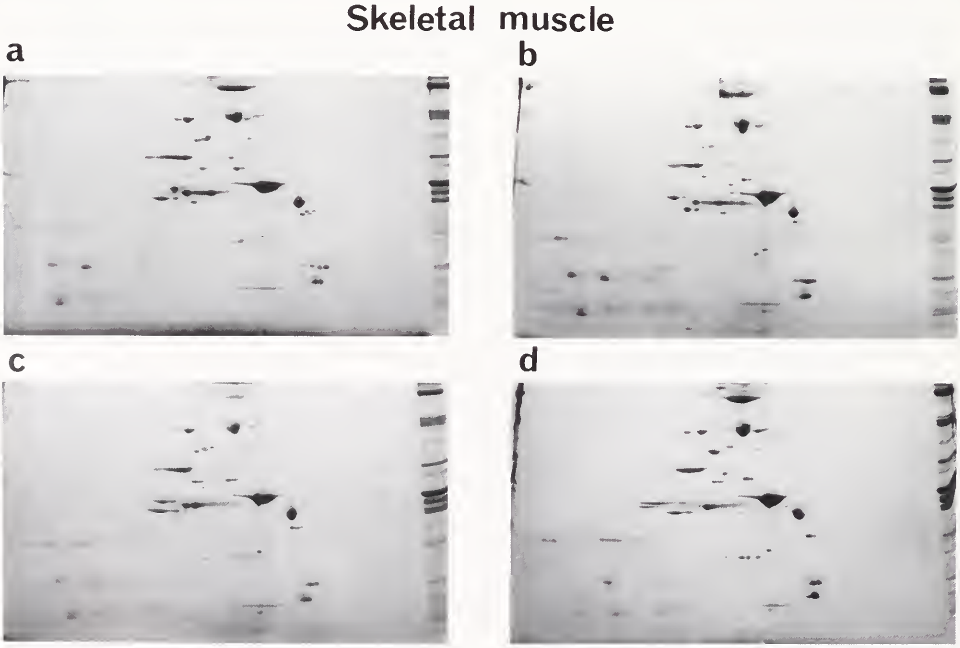

FIGURE 4. The typical two-dimensional electrophoresis patterns of skeletal muscle. a, *Tachypleus tridentatus;* b, *Tachypleus gigas;* c, *Carcinoscorpius rotundicauda;* d, *Limulus polyphemus.* The reference for total supernatant components is shown on the right-hand end.

TABLE V

*Similarity among the four species based on presumed protein groups*

| Combination | Cardiac muscle $N_x$ | Cardiac muscle $N_y$ | Cardiac muscle F | Skeletal muscle $N_x$ | Skeletal muscle $N_y$ | Skeletal muscle F |
|---|---|---|---|---|---|---|
| *T. tridentatus* and *T. gigas* | 100 | 100 | 0.60 | 95 | 97 | 0.65 |
| *T. tridentatus* and *C. rotundicauda* | 100 | 98 | 0.61 | 95 | 99 | 0.59 |
| *T. gigas* and *C. rotundicauda* | 100 | 98 | 0.63 | 97 | 99 | 0.61 |
| *T. tridentatus* and *L. polyphemus* | 100 | 88 | 0.38 | 95 | 85 | 0.46 |
| *T. gigas* and *L. polyphemus* | 100 | 88 | 0.32 | 97 | 85 | 0.47 |
| *C. rotundicauda* and *L. polyphemus* | 98 | 88 | 0.32 | 99 | 85 | 0.42 |

Calculation of the similarity (F) is according to Aquadro and Avise (1981).

similar to each other. This supports the biochemical, embryological, and genetical evidences which show that differences among the three Asian species are not sufficient to justify division into two genera (Sekiguchi and Sugita, 1980; Shishikura *et al.*, 1982; Srimal *et al.*, 1985). However, our phenetic analyses for cardiac and skeletal muscles do not give new insight into the problem of whether *Tachypleus tridentatus* is more similar to *Carcinoscorpius rotundicauda* than to *Tachypleus gigas.*

On the other hand, our cladistic analysis shows that *T. tridentatus* may be more closely related to *C. rotundicauda* than to *T. gigas,* thus supporting the biochemical, embryological, and genetical view cited above. However, only 4 of the 93 cardiac and skeletal muscle protein groups examined indicate these relationships. This number probably is too small to comfortably deduce phylogenetic relationships. Therefore, we must be careful before drawing a conclusion from our data. Nevertheless, all cases, in which derived character states are shared, suggest a sister-group relationship between *T. tridentatus* and *C. rotundicauda.*

The ambiguity of phylogenetic relationships among the Asian species of horseshoe crabs suggested by our phenetic and cladistic analyses raises doubts about the applicability of two-dimensional electrophoresis for phylogenetic studies. Some concerns for this method have been recognized by McConkey (1982). The most important is that two-dimensional electrophoresis gives information only about isoelectric points and molecular weights of proteins and tells nothing about their functions, even though proteins with similar points and weights may have the same functions. The second major concern is that adjacent or partially overlapping large spots may appear as one spot. This may be addressed by using varing amounts of proteins (*i.e.*, volumes of supernatants) for two-dimensional analysis.

The use of two-dimensional gel electrophoresis in phylogenetic studies has been more controversial than that of conventional (native gel) electrophoresis. Aquadro and Avise (1981) have shown that genetic distances among rodents, revealed by two-dimensional electrophoresis, is about half of those shown by native gel electrophoresis. In their opinion, kinds of proteins detected by the two methods may be different, and it is not clear whether the charge-equivalent substitutions of amino acids can be detected by two-dimensional electrophoresis, since it involves denaturing of proteins.

Studies of genetic heterozygosity with two-dimensional electrophoresis have also shown lower estimates than those by native gel electrophoresis (Brown and Langley, 1979; McConkey *et al.*, 1979; Walton *et al.*, 1979; Racine and Langley, 1980). These authors have pointed out that the native gel method is more sensitive both to conformational and post-translational protein differences, and to variable proteins (*i.e.*, enzymes) than the two-dimensional method. Enzymes also vary, however, including less variable ones that are substrate-specific, are involved in energy metabolism, or utilize intracellular substrates (Gillespie and Kojima, 1968; Johnson, 1973; Cohen *et al.*, 1973). Therefore, even enzymes detected by native gel electrophoresis can be divided into two types (variable or specific). Furthermore, Brown and Langley (1979) have shown that heterozygosity estimates obtained by native gel electrophoresis of specific enzymes are smaller than those from variable enzymes, but are not different from those by two-dimensional electrophoresis. The higher heterozygosity from native gel electrophoresis can be attributed to preferred use of easily assayed variable enzymes. Two-dimensional electrophoresis, on the other hand, detects more numerous proteins that may be conservative (less variable) due to physiological constraints as so specific enzymes are.

Multimeric proteins are less variable than monomeric ones (Zouros, 1976; Harris *et al.*, 1977; Ward, 1977). Likewise, structural ribosomal proteins show less variation than variable enzymes (Berger and Weber, 1974; Bucknall *et al.*, 1975). Edwards and Hopkinson (1980) and McConkey (1982) suggested that such less variable proteins are involved in macromolecular interactions, such as vital protein-protein interactions and binding between protein and lipid/carbohydrate moieties. Because of their conformational constraints, these proteins, which are parts of complex structures, are more conservative than those which are solitary or parts of simple structures. We believe that most proteins detected by two-dimensional electrophoresis are those associated with complex structures (as also suggested by McConkey).

Therefore, native gel electrophoresis of variable enzymes is advantageous for detection of genetic differences among organisms. However, amino acid or DNA sequencing may be more preferable and informative when examining such a particular and defined class of proteins. If the goal is a comprehensive and balanced view of genetic differences among organisms, then the use of two-dimensional electrophoresis seems to be advantageous, since this procedure allows examination of at least some representatives of all classes of proteins. Although the two-dimensional electrophoresis method has some difficulties and detects prevalent less variable proteins (McConkey, 1982), it is a useful method which permits us to examine many kinds of proteins at one time.

Ayala *et al.* (1974) and Avise (1975) showed that native gel electrophoresis provides a valuable tool for systematic examination of genetic divergence among closely related species or congeneric species, as well as intraspecific populations. However, because of its high sensitivity for genetic differences, this method is not useful for determining relationships at taxonomic levels above species. In this case, we must use a limited number of very specific (less variable) enzymes such as lactate dehydrogenase (Shaklee and Whitt, 1981). Based on available data (for example, see Table 1 in Aquadro and Avise, 1981) and our results, the two-dimensional electrophoresis method permits us to estimate and evaluate genetic differences at higher taxonomic levels. Furthermore, Aquadro and Avise (1981) showed that genetic distance ranks, obtained by two-dimensional electrophoresis, are highly concordant with ranks of taxonomic levels. Therefore, we suggest that this technique does provide a valuable tool for systematics.

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# EGG PRODUCTION BY SAND CRABS (*EMERITA ANALOGA*) AS A FUNCTION OF SIZE AND YEAR CLASS (DECAPODA, HIPPIDAE)

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## ABSTRACT

The relationship between egg number, size, and year class was determined for sand crabs (*Emerita analoga*) collected at three California sites, including two different dates for two of those sites. Size frequency distributions of sand crab population samples collected in 1982 from San Clemente, Goleta (2 dates), and Pismo Beach (2 dates) were separated into three modal size/year classes before plotting the size and egg number data and calculating regressions for egg number as a function of size. The slope of the regressions for within year class data generally decreased with increase in age. Both seasonal and locality differences were found in comparisons of data for individual year classes. The technique can be used to compare egg production between female crabs of similar ages between sites and/or dates.

## INTRODUCTION

Female crustaceans generally produce more eggs as they grow larger (*e.g.,* Barnes and Barnes, 1968; Van Dolah and Bird, 1980; Hartnoll, 1985). Other animals with indeterminate growth, such as fish (*e.g.,* DeMartini and Fountain, 1961; Bagenal, 1967; Macgregor, 1968; Moe, 1969; Dietrich, 1979; Hunter and Macewiez, 1980; Love and Westphal, 1981; Baltz and Knight, 1983; see also the earlier listing in Altman and Dittmer, 1972), also produce greater numbers of eggs as they grow larger.

The fact that eggs are brooded externally and are thus visible in most crustaceans has led to a considerable amount of research on size-related egg production in that group (*e.g.,* isopods, Paris and Pitelka, 1962, and Lawlor, 1976a, b; mysids, Mauchline, 1973; prawns, Wickens and Beard, 1974; fairy shrimps, Daborn, 1975; barnacles, Barnes and Barnes, 1968, and Hines, 1976; amphipods, Von Dolah and Bird, 1980; hermit crabs, Bertness, 1981; lobsters, Ennis, 1981; krill, Denys and McWhinnie, 1982; brachyurans, Hines, 1982; copepods, Carter *et al.,* 1983; and tanner crabs, Somerton and Meyers, 1983).

Egg production by hippid crabs, which are easily collected and occur throughout the world (Efford, 1976), has received much attention (*e.g.,* Osorio *et al.,* 1967; Efford, 1969; Eickstaedt, 1969; Subramoniam, 1977, 1979; Diaz, 1980). Estimates of hippid egg production have usually been done as a part of life history studies.

While studying reproductive patterns of the common sand crab (*Emerita analoga*) living near a power plant (*e.g.,* Auyong, 1981; Siegel and Wenner, 1984), it became apparent that the overall reproductive output of a population might be impaired if sand crabs were under environmental stress. However, the published data

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on *E. analoga* egg production were not sufficient for estimating reproductive output and were frequently inconsistent (*e.g.*, see Eickstaedt, 1969, and Efford, 1969).

The present report summarizes the results of a study of the relationship between size, age, and egg production in three geographically separated populations of the common sand crab at different times during the reproductive season, as clarified by a modal size class (year class) analysis (*e.g.*, Harding, 1949; Cassie, 1954; Wenner and Fusaro, 1979).

## MATERIALS AND METHODS

The analysis of egg production and size/year class relationships was based upon ovigerous sand crabs of all available sizes collected in 1982 from each of three California locations, two of which were in the Southern California Bight (San Clemente and Goleta). Fifty-nine ovigerous crabs were collected on 3 August 1982 from San Clemente State Park beach. Two collections were made at Goleta Beach Park; on 25 August 1982, 83 ovigerous crabs were collected, and on 13 October (49 days later, at the end of the reproductive season) 52 more crabs were collected in that area. Two collections were also made at the Pismo Beach pier, on 17 May 1982 (51 ovigerous crabs) and 119 days later on 13 September (25 crabs).

Crabs were initially measured with a graded sieve (Wenner *et al.*, 1974) to the nearest 0.5 mm carapace length (if less than 17.5 mm CL) or to the nearest 1 mm CL (if more than 17.5 mm CL). The size frequency distribution data for all female crabs (ovigerous and non-ovigerous) were then separated into component modal groups (*e.g.*, Harding, 1949; Cassie, 1954; Wenner and Fusaro, 1979; Siegel and Wenner, 1985). The use of probability graph paper for breaking size frequency distributions into constituent modal size/year classes is appropriate because sand crab laboratory growth rates were correlated with year class modes obtained by analyses of changes in field population structure through time (Siegel and Wenner, 1985).

When possible, 5 crabs with newly extruded eggs (stages 2–4 of Boolootian *et al.*, 1959; Eickstaedt, 1969) were chosen for egg counts from each of the 0.5 or 1.0 mm size classes. Crabs with eggs still attached were preserved in a mixture of 30% seawater, 30% ethanol, 30% acetone, and 10% glycerol, a mixture in which the egg diameters remained constant until counting.

Prior to estimating egg number, each preserved crab was measured with calipers to the nearest 0.05 mm carapace length (CL) and washed gently in fresh seawater. Eggs were then removed from the pleopods, checked under the microscope for developmental stage, examined for parasite presence, and placed in an open-ended pipette with a 250 $\mu$ nylon screen glued to the bottom to permit water displacement. Known numbers of eggs added to the submerged pipette permitted calculation of a calibration curve, allowing egg number to be estimated by volume in a technique similar to that used by Diaz *et al.* (1983). Eggs were counted directly if they numbered fewer than 1000.

Data points were first ascribed to the appropriate size/year class, after determination by prior modal size class analysis. The data were then plotted in semi-log transformation to obtain regressions for egg number as a function of size. The sizes of crabs within year classes were compared between sites using one-way ANOVA and Tukey-type multiple comparison tests. ANCOVA was not used, because it was not appropriate for the entire data set due to the fact that the year classes, after separation, had non-overlapping ranges for the covariate size. The relationship between size and egg number was compared between year classes at each site and date with multiple comparison of slopes, by use of a Tukey-type comparison (Zar, 1984).

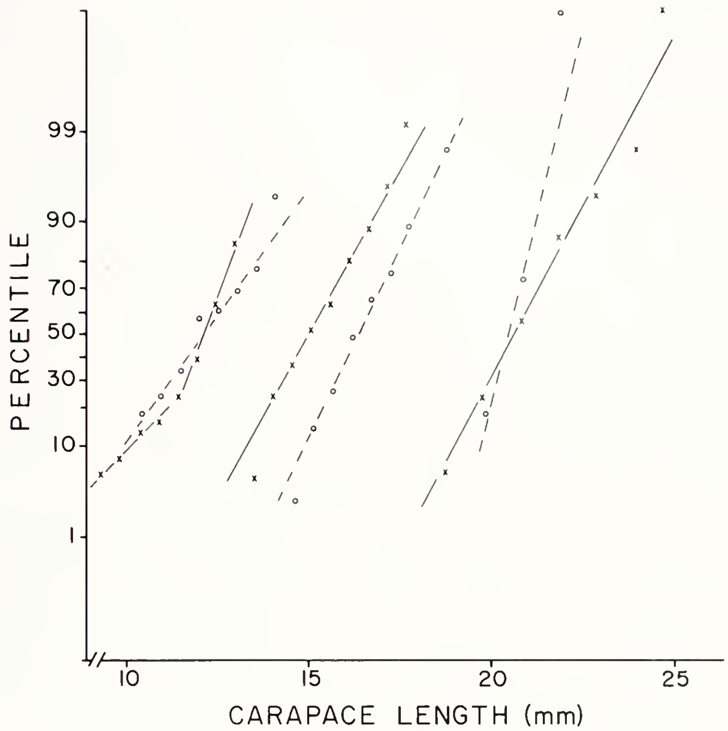

FIGURE 1. Modal size class breakdown for sand crab population structure in 1982 at San Clemente on 3 August 1982 (open circles) and at Goleta on 25 August 1982 (×).

## RESULTS

### *Modal size class analysis and egg number*

The modal size class analysis of the frequency distributions yielded similar results for Goleta and San Clemente (the two sampling sites in the Southern California Bight), with three distinct year classes apparent at each location (Fig. 1; see also Siegel and Wenner, 1985). The overall pattern of egg number as a function of size at those two sites was also similar for the first two year classes (Figs. 2, 3; Table I), but egg production by the few third year crabs which were found at San Clemente was highly variable.

The slope of the line depicting egg number as a function of size was quite steep for first year and less so for second year crabs from the San Clemente sample. Since third year ovigerous crabs were scarce and the egg numbers highly variable (Fig. 2), the regression line for that mode was omitted from the figure (included in Table I).

The regression of the 25 August Goleta data for egg number as a function of size for each year class yielded three corresponding lines with their slopes again decreasing with age and size (Fig. 3; Table I). The egg number data for third year Goleta crabs were tightly clustered and consistent compared to the high scatter in egg number for third year crabs from the San Clemente site.

Whereas a high percentage of first year crabs produced eggs at both southern sites, only one first year ovigerous crab was found in the 13 September Pismo Beach sample

TABLE I

*Size and egg number relationships for modal size/age classes of female sand crabs* (Emerita analoga) *collected from three California sites in 1982*

| | | Mode I | Mode II | Mode III |
|---|---|---|---|---|
| San Clemente | Formulas | $\log y = 0.19x + 0.49$ | $\log y = 0.09x + 2.14$ | $\log y = 0.12x + 1.23$ |
| (3 Aug.) | | ($n = 24$; $r = 0.85$) | ($n = 29$; $r = 0.77$) | ($n = 6$; $r = 0.56$) |
| | Mean size (mm CL) | 13.09 ± 1.03 | 16.85 ± 1.20 | 19.88 ± 0.97 |
| | Mean egg # (±SD) | 1193 ± 626 | 4128 ± 1237 | 4782 ± 2149 |
| Goleta | Formulas | $\log y = 0.20x + 0.62$ | $\log y = 0.12x + 1.65$ | $\log y = 0.04x + 3.01$ |
| (25 Aug.) | | ($n = 25$; $r = 0.90$) | ($n = 38$; $r = 0.87$) | ($n = 20$; $r = 0.73$) |
| | Mean size (mm CL) | 12.17 ± 0.91 | 15.64 ± 1.16 | 20.40 ± 1.43 |
| | Mean egg # | 1109 ± 449 | 3069 ± 1036 | 6808 ± 1216 |
| Goleta | Formulas | $\log y = 0.04x + 0.13$ | $\log y = 0.12x + 1.19$ | $\log y = 0.05x + 2.46$ |
| (13 Oct.) | | ($n = 13$; $r = 0.10$) | ($n = 25$; $r = 0.48$) | ($n = 14$; $r = 0.33$) |
| | Mean size (mm CL) | 13.38 ± 0.68 | 16.64 ± 0.90 | 22.00 ± 1.35 |
| | Mean egg # | 567 ± 317 | 1510 ± 703 | 11,900 ± 3650 |
| Pismo | Formulas | | $\log y = 0.074x + 2.23$ | $\log y = 0.06x + 2.44$ |
| (17 May) | | | ($n = 23$; $r = 0.83$) | ($n = 28$; $r = 0.69$) |
| | Mean size (mm CL) | | 21.23 ± 1.77 | 27.93 ± 1.55 |
| | Mean egg # | | 4400 ± 1450 | 11,900 ± 3650 |
| Pismo | Formulas | | $\log y = 0.07x + 2.35$ | $\log y = 0.02x + 3.67$ |
| (13 Sept.) | | ($n = 1$) | ($n = 19$; $r = 0.76$) | ($n = 5$; $r = 0.34$) |
| | Mean size (mm CL) | 17.2 mm | 23.47 ± 1.40 | 29.02 ± 1.48 |
| | Mean egg # | 1513 | 9600 ± 2490 | 17,160 ± 3350 |

(Fig. 4; Table I). Pismo Beach crabs in the second and third year classes produced considerably more eggs than those crabs in the same year classes from Goleta (25 August) and San Clemente (3 August) beaches. (The modal size class separation for crabs collected on 17 May from Pismo Beach appears in Siegel and Wenner, 1985; the three year class modes were very distinct at Pismo Beach.) The size range and egg production values obtained for second and third year crabs at Pismo Beach essentially did not overlap data for second and third year crabs either from San Clemente or from Goleta Beach.

Within each year class the ovigerous female crabs were different in size at Pismo (September), Goleta (August), and San Clemente (ANOVA: year 1, $P < 0.0014$, year 2, $P < 0.001$, year 3, $P < 0.001$). The differences were statistically significant in all comparisons (Tukey multiple comparisons: $P < 0.001$), except in the comparison of third year female crabs for Goleta (August) and San Clemente (n.s.). (The data for egg numbers were not compared between sites for each of the year classes in this study due to the appreciable size differences found; see methods.)

Comparisons of the slopes of the regressions of size and egg number for each year class at each sampling date and site revealed significant differences in slope between year classes for first and second year female crabs at San Clemente ($P < 0.005$) and all three year classes in August at Goleta ($P < 0.001$). All other slope comparisons yielded differences that were not significant.

The difference in mean size of crabs in the equivalent year class modes, both between year classes at the same time of year and between collecting dates in 1982, agreed with earlier results gathered with respect to the amount of growth expected for a year class mode within the season and between years (*e.g.*, Siegel and Wenner, 1985).

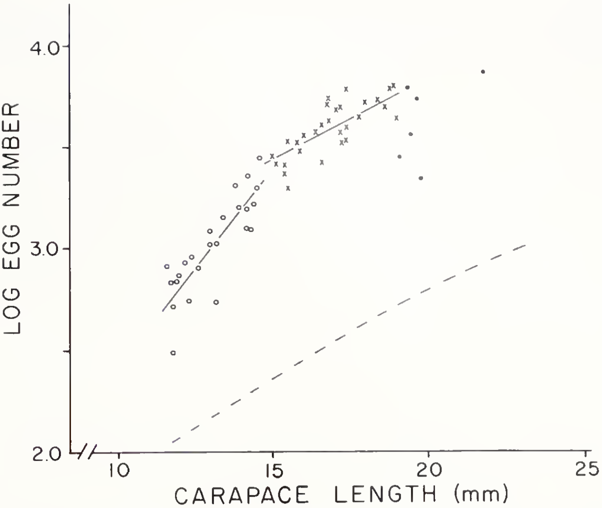

FIGURE 2. Egg production as a function of size for the San Clemente State Beach sample of 3 August 1982. Open circles represent first year crabs, the × marked individuals are second year crabs, and the closed circles represent data for third year crabs. The broken line was extracted from a plot published by Efford (1969); see text.

The mean number of eggs produced within each year class decreased between the two sampling dates (25 August and 13 October) for Goleta crabs (Table I, Student's *t*-test; $P < 0.001$), despite the significantly larger mean size of female crabs in the

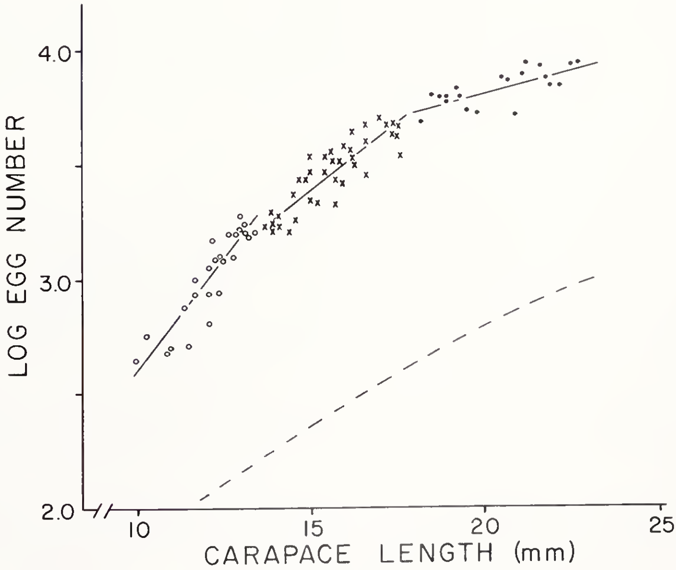

FIGURE 3. Egg production as a function of size for Goleta sand crabs on 25 August 1982. Symbols and broken line are the same as for Figure 2.

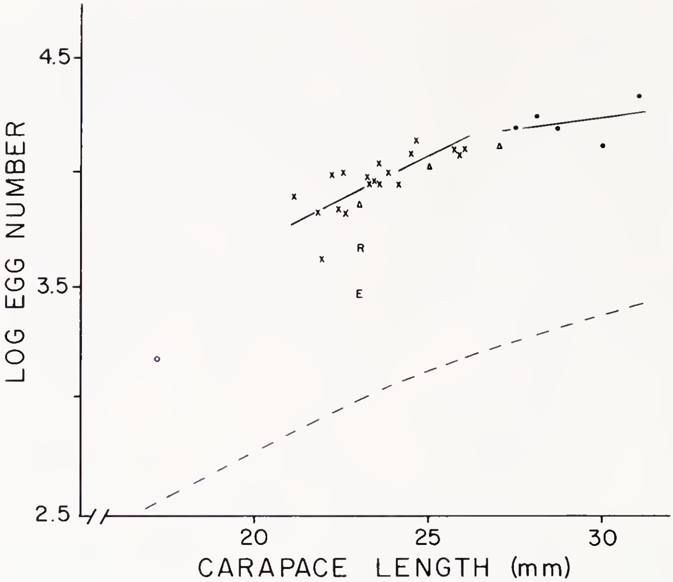

FIGURE 4. Egg production as a function of size for Pismo Beach crabs on 13 September 1982. Most symbols and broken line are as for Figures 2 and 3. The three triangles represent three data points from Eickstaedt (1969); the *R* and *E* symbols represent data points for Renata and El Tabo, Chile, South America (Osorio *et al.*, 1967).

October sample (Student's *t*-test; $P < 0.005$). By contrast, the mean number of eggs produced within each year class at Pismo (Table I, Student's *t*-test; $P < 0.005$) increased between the two sampling dates (17 May and 13 September), also while mean size of crabs increased. The time between samples was sufficiently long for full brood development in both cases; those data thus represent separate times of egg extrusion for each population sample. (Incubation time is less than 40 days; *e.g.*, Eickstaedt, 1969.)

No egg mass parasites were found in any of the samples.

### *Comparison of results with those obtained by other workers*

Figures 2, 3, and 4 also contain a smooth broken line, derived from a straight line plot published by Efford (1969; Fig. 6). (His data were obtained from samples taken at various times during the reproductive season and from 20 different localities between Baja California, Mexico, and British Columbia, Canada.) In his figure, Efford had fitted a straight line on a log-log plot with unequal scaling for the two axes. After re-plotting on either standard log-log or on semi-logarithmic axes, the line becomes curved. The comparison shown here (Figs. 2–4) indicates that Efford's egg number estimates were approximately one-tenth the values obtained in the present study (Figs. 2–4).

As an additional comparison, egg count estimates are included in Figure 4 from Eickstaedt (1969) for 3 crabs of 23, 25, and 27 mm carapace length collected on 25 June 1968 in the Monterey area. Those values obtained by Eickstaedt correspond with the values we obtained for similar-sized second and third year crabs at Pismo Beach during 1982. Because his values differed so much from values obtained by

Efford for the same size crabs collected from that same area, Eickstaedt counted, rather than estimated, egg number in other clutches from 4 crabs of equivalent sizes collected in the same area on 10 June 1969 and obtained results similar to his earlier estimates.

Egg count results published by Osorio *et al.* (1967) for South American populations of *Emerita analoga* are also included in Figure 4; those counts were approximately 4–5 times greater than reported by Efford for similar-sized crabs. The mean number of eggs for El Tabo and Renaca crabs were significantly lower than our estimates in 9 comparisons out of 12 when compared to egg numbers for Goleta crabs (Student's *t*-test, $P < 0.05$). The animals within given year classes were larger at El Tabo and Renaca than those at Goleta, but they produced fewer eggs.

## Discussion

While many papers on crustacean biology have included data on the number of eggs produced as a function of size (*e.g.*, see Introduction), few have addressed the question of just how much variation can exist as a consequence of their indeterminate growth and multiple broods through time. One of the notable exceptions is the classic study of Paris and Pitelka (1962) on terrestrial isopods (*Armadillidium vulgare*). They found that the number of eggs produced by those isopods varied as a function of size/age class, season, and locality.

Marine crustaceans generally present a more formidable problem for egg production studies than terrestrial isopods, since year classes may be difficult to ascertain. Hippid crabs may be an exception. They have served as material for studies of population structure and egg production in the marine habitat (*e.g.*, Osorio *et al.*, 1967; Barnes and Wenner, 1968; Cox and Dudley, 1968; Efford, 1969; Eickstaedt, 1969; Subramoniam, 1977; Diaz, 1980; and Perry, 1980), but only to a limited extent.

Several features of hippid crab biology render them suitable for more full-scale investigations of population structure and egg production. They are hardy, often occur in great numbers (*e.g.*, Efford, 1976), can be collected easily, can be measured readily (Wenner *et al.*, 1974), and survive well in quality seawater systems. They live at least three years (3 years maximum for *Emerita analoga;* Siegel and Wenner, 1985), permitting separation of population structure into modal size classes (*e.g.*, Wenner and Fusaro, 1979), which appear to be year classes (Siegel and Wenner, 1985).

This study of egg production in *Emerita analoga* revealed that the relationship between egg number and length or width can be analyzed in conjunction with prior separation of the size frequency distribution into modal size/age classes. The modal year class separation permitted a more detailed analysis of egg production as a function of size than has prevailed before (*e.g.*, Efford, 1969; Eickstaedt, 1969). Additional information was provided by the modal separation approach, opening the way for quantitative comparisons of variation within season, between years, and between localities.

After data on egg number as a function of size had been plotted in all conventional manners, it was apparent that a power function was present and that the semi-log plot was an appropriate representation. That is because the linear measurement of either length or width, as is common in crustacean studies, is being compared to the number of eggs in a three-dimensional egg mass. In addition, a semi-logarithmic plot is more convenient for retrieving data than either the log-log plot or any plot involving the cube of the animal's length or width.

For the one population which had a full complement of three year classes of ovigerous crabs (Goleta), the slope of the regression line decreased with the size/age class of the animals. Without the modal size class breakdown, one could conclude that the relationship between egg production and size was curvilinear on a semi-log plot. However, the technique of splitting modes and analyzing egg production within each mode, as illustrated here, permits a biologically meaningful comparison of egg production by year classes between seasons, years, and localities.

Third year crabs were scarce at the San Clemente site, and the variation in the number of eggs produced by those few crabs was great. Both Auyong (1981) and Wenner (1982) reported a marked scarcity of third and sometimes even second year crabs in that area, especially at sites further south which were closer to the San Onofre Nuclear Generating Station. Those results suggested a failure at overwintering, which could be related to the disruption of egg production (Siegel and Wenner, 1984) in that area.

The modal separation approach can permit a resolution of apparent discrepancies in egg production results found for different localities and in different seasons. First year crabs apparently produce a single clutch of eggs at sites in the Southern California Bight before the end of summer (*e.g.*, Cox and Dudley, 1968; Siegel and Wenner, 1985). However, the time at onset of egg production of first year crabs may vary widely (see Cox and Dudley, 1968; Wenner *et al.*, 1985).

Crabs from Pismo Beach in Central California do not usually produce eggs until their second year on the beach (Fig. 4 and unpub. data), which may explain their larger size at sexual maturity in that region (Wenner *et al.*, 1985). The larger size at the onset of sexual maturity at Pismo Beach may be related both to a slower rate of ovarian development in cooler waters and to a faster growth rate brought about by greater food availability. Annala *et al.* (1980) also reported an inverse relationship between temperature and size at maturity in spiny lobsters (*Jasus edwardsii*) in New Zealand waters.

Second and third year crabs at Pismo Beach were larger and produced more eggs than their counterparts at Goleta and San Clemente. Other investigators have found such variation in crustacean egg production at different locations for similar-sized animals without separating population structure into constituent modal year classes (*e.g.*, Barnes and Barnes, 1968; Steele and Steele, 1975; Hill, 1977; Jones and Simons, 1983). Osorio *et al.* (1967) also found different egg numbers for sand crabs (*Emerita analoga*) of equivalent year classes at two sites in South America.

The seasonal differences found in egg numbers at Goleta and Pismo Beach were striking and similar to those found by Eickstaedt (1969) in a more limited study. Paris and Pitelka (1962) also reported such differences during the season in their study of isopod egg production. If such seasonal differences are common in animals which produce multiple broods during each reproductive season, calculation of life tables becomes a far more difficult matter than the traditional textbook examples would indicate.

Efford (1969) and Eickstaedt (1969) reported widely different values for the number of eggs produced by equivalent-sized crabs. Eickstaedt's concern about that discrepancy and subsequent direct counts (rather than estimates) indicated that Efford had erred. A replotting of Efford's regression line on the displays obtained in this study reveal that the values reported by Efford were approximately one order of magnitude lower than those obtained either by Eickstaedt or in this study. Eickstaedt (1969; pp. 86, 87) attributed the difference in estimated numbers to Efford's apparent failure to check his estimated values by direct counts.

An accurate estimate of the number of eggs produced by females in populations can be used to assess the effects of different environmental conditions within or between habitats (*e.g.*, Wenner and Fusaro, 1979). That measure includes the number of eggs produced per given size or age of animal and the total number of eggs produced per year by a representative population sample (*e.g.*, the number of eggs per representative thousand females). For animals with indeterminate growth, especially for crustaceans which possess no indication of age and which may produce multiple broods within a season, the task of estimating the number of eggs for a representative population sample and/or the reproductive output of a population requires a detailed analysis.

The results of this study indicate that a separation of size frequency distributions into constituent year classes can be done before one examines egg production as a function of size. This modal separation technique provides more information than the traditional method of analyzing egg production as a function of size without regard for age. Application of the technique can enhance seasonal, geographical, and environmental comparisons of variation in egg production, as well as aid in estimating total reproductive output.

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# POLYKARYON FORMATION BY *MERCENARIA MERCENARIA* HEMOCYTES

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## ABSTRACT

Examination of *Mercenaria mercenaria* hemocyte preparations on glass coverslips showed that adherent, macrophage-like cells tended to form multinucleated giant cells morphologically similar to classical Langhans cells and foreign body inflammatory giant cells, as seen in vertebrates. The frequency of giant cell formation varied from clam to clam, possibly reflecting each animal's physiological state, as influenced by pollutant exposure, other environmental stressors and disease. Treatment of the hemocytes with concanavalin A promoted hemocyte aggregation and increased the rate of giant cell formation. Attempts to increase polykaryon formation by *in vivo* or *in vitro* phagocytic stimulation of the hemocytes were generally unsuccessful.

## INTRODUCTION

Although the formation of polykaryons in response to inflammation has been frequently seen in vertebrates, little is known about the process in invertebrates. Giant cell formation in lower vertebrates was reported as early as 1926 by Lewis and Lewis in hanging-drop blood cell cultures. There are several reports of giant cells *in vivo* in mollusks; these cells were observed during postmortem examination of oysters by Sparks and Pauley (1964), and also associated with allografts and xenografts in gastropods by Cheng and Galloway (1970). These cells, designated "macrocytes," were thought to arise from fusion of mature granulocytes (Cheng, 1981). This study reports the *in vitro* formation of giant cells in hemocyte monolayer preparations from the bivalve *Mercenaria mercenaria.*

Multinucleated giant cells (MGC) can be classified as inflammatory foreign body cells (FBC), or Langhans cells (LC), based on morphological criteria (Chambers and Spector, 1982). Foreign body type polykaryons have numerous nuclei randomly distributed throughout their cytoplasm. In contrast, Langhans cells have nuclei arranged in circular or semi-circular patterns near the periphery of the cell. There appear to be many intermediate cell forms between the foreign body and Langhans types.

The exact manner by which giant cells arise has been the subject of controversy. It was proposed that karyokinesis in the absence of cytokinesis was responsible for the formation of the giant cell syncytium (Virchow, 1858). Borrel (1893) postulated that cell fusion was the primary phenomenon behind the formation of polykaryons. Current evidence favors the cell fusion theory (Mariano and Spector, 1974; Murch

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Abbreviations: con A = concanavalin A; FBC = inflammatory foreign body cells; LC = Langhans cells; MGC = multinucleated giant cells; SSW = sterile seawater.

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*et al.,* 1982). Observations made during the present study also tend to support the cell fusion model in *Mercenaria.*

## MATERIALS AND METHODS

Commercially obtained *Mercenaria* were maintained at 8°–12°C in 30 gallon recirculated artificial seawater aquaria.

A hypodermic needle was inserted into the posterior adductor muscle blood sinus to collect hemolymph samples. Hemolymph drops were placed on ethanol-cleaned coverslips, and the cells were allowed to adhere for 30–60 minutes in a moist chamber at room temperature. Nonadherent cells were gently washed off with sterile seawater (SSW) and the adherent cells bathed in medium. The medium consisted of 18 ml of cell-free (1500 × *g,* 15 min) hemolymph, 17.5 ml buffered seawater (pH 7, 0.05 *M* HEPES), 0.5 ml antibiotic solution (10,000 U/ml penicillin and 10 mg/ml streptomycin), and 40 mg of glucose. This medium was sterilized by ultrafiltration (0.45 m pore size) and stored at 4°C. In long-term studies, the medium covering the hemocytes was changed daily. The use of buffered, antibiotic-containing medium was essential to prevent a subsequent overgrowth of contaminating bacteria. The cell monolayer preparations were rinsed with SSW, fixed in 10% formalin in SSW, washed, stained with hematoxylin, dehydrated by passage through an ethanol-water gradient, stained with eosin, cleared in xylene, and mounted on slides. Morphology was studied by brightfield and/or phase contrast microscopy.

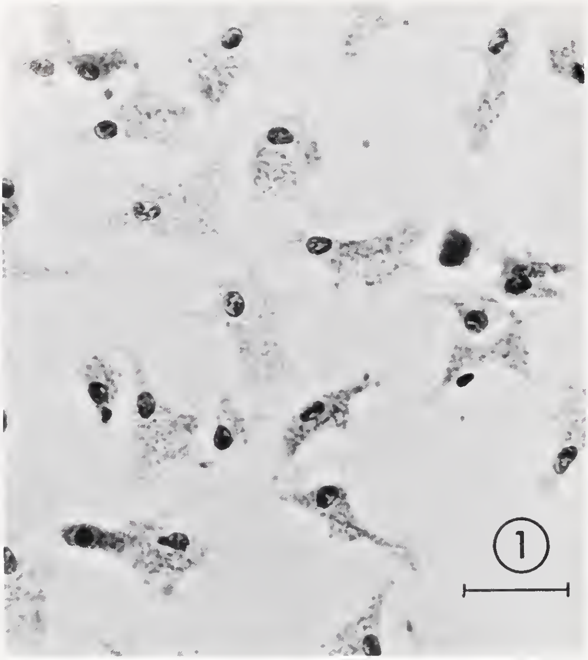
Attempts were made to induce giant cell formation *in vitro* by adding various concentrations of foreign particulates to the medium. The particulates tested included yeast cells, latex beads, graphite, titanium dioxide, carbon, iron, aluminum, brass, or silica. Yeast cells, carbon, or the above-mentioned metal dust suspensions were also injected into the adductor muscle blood sinus in an attempt to induce polykaryon production *in vivo.* Specific details of dosages and times for these studies will be given in the Results section. The effects of the addition to the medium of the plant lectin concanavalin A (Sigma Chemical Company) on giant cell formation by clam hemocytes were also determined.

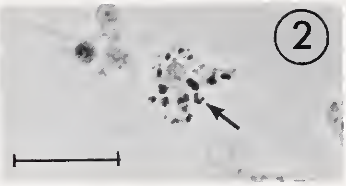
## RESULTS

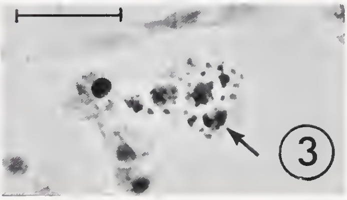
*Mercenaria* hemocytes adhered rapidly and firmly to the glass substrate, and remained in an evenly dispersed cell monolayer for at least an hour (Fig. 1). Small cells containing irregular yellow-brown granules were seen in many of the cell preparations (Figs. 2, 3). The granules in these cells did not resemble the usual inclusions seen in *Mercenaria* granulocytes, or other hemocyte classes. Their relationship to the inclusions described in the so-called brown or serous excretory cells described by Ruddell and Wellings (1971) and Moore and Lowe (1977) remains to be ascertained.

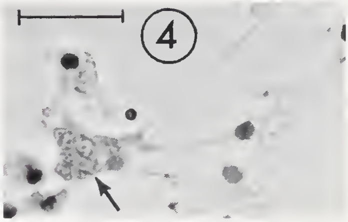
Small MGC were present in some hemocyte preparations as soon as one hour in culture. These cells usually resembled small inflammatory giant cells; Figure 4 shows such a cell having five central nuclei from a 3 h culture. The nuclei in FBC usually appeared slightly swollen with light-staining, diffuse chromatin. The FBC continued to increase in size, until they contained numerous, randomly oriented nuclei (Fig. 5). Irregular yellowish granules, similar to those noted in small cells (Figs. 2, 3) prior to MGC formation, could be observed in the region of the nuclei of many FBC.

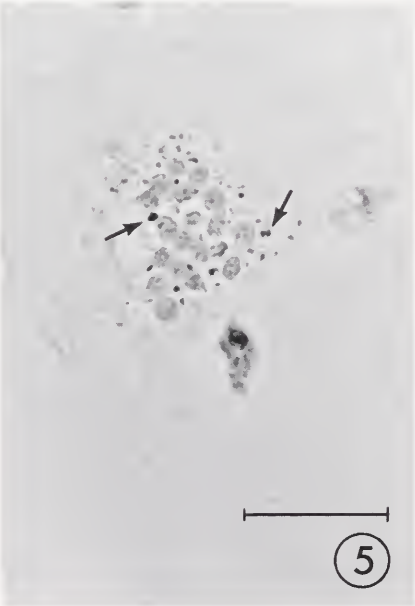
After about 6–8 h of maintenance, some of the hemocyte cultures contained small Langhans cells with characteristic ring-like arrangements of 8–20 nuclei (Fig. 6). The LC continued to develop for 24–48 h until they contained 50 to >100 nuclei arranged in concentric circles near the periphery of the cell (Fig. 7).











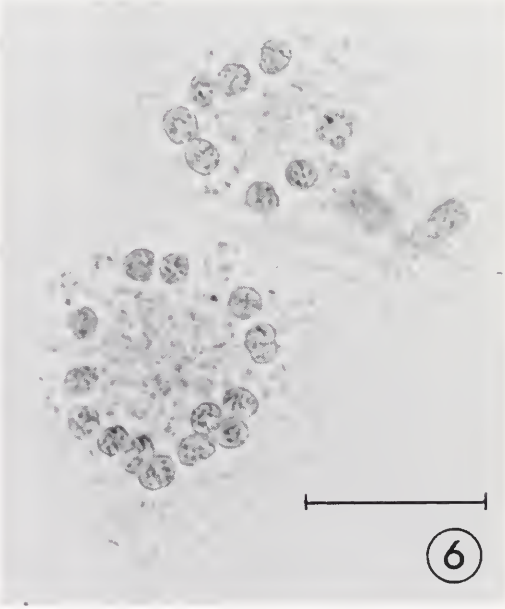


FIGURE 1. Typical appearance of hemocyte monolayer 1 h after adhesion to the glass substrate.

FIGURE 2. A hemocyte containing numerous irregular yellow granules (arrow).

FIGURE 3. Hemocyte with large ($2 \times 5$ $\mu$m) clumped yellow-brown inclusions (arrow).

FIGURE 4. Early inflammatory foreign-body type cell after 3 h culture, arrow indicates one of five closely packed nuclei.

FIGURE 5. Typical FBC after 5 h in culture containing 18 nuclei; one of many yellow-brown granules, similar to those in Figures 2 and 3, is indicated by the arrow.

FIGURE 6. Langhans type cells in a 6 h hemocyte culture. Scale bar = 20 $\mu$m for Figures 1–6.

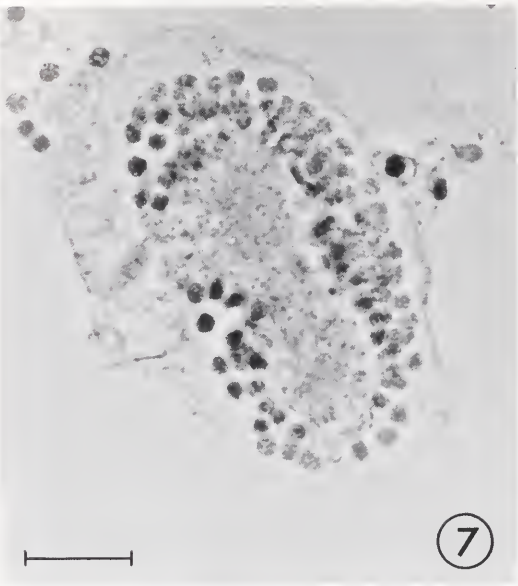
The FBC also continued to enlarge during 24–48 h in culture. In these preparations, FBC are often connected by numerous cytoplasmic strands (Fig. 8). The more mature FBC frequently contained foreign material such as amorphous masses (Fig. 8), yellow-brown particles (Fig. 9) or cell-like structures (Fig. 10). The cell-like inclusions were typically surrounded by a conspicuous vacuole; however, the yellow granules and amorphous material were not usually enclosed by vacuoles. Multinucleated giant cells of either kind were rarely seen in >48 h hemocyte monolayer cultures; possibly they lysed or became less adherent, and were lost during medium changes.

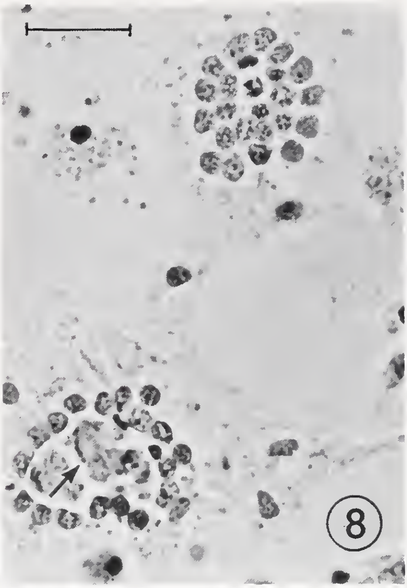
Giant cell formation was a highly variable phenomenon in *Mercenaria.* Groups of 10–24 clams were examined at a given time; usually 50–60% of the individual organisms per batch would yield MGC-producing hemolymph samples, but this number could be as low as 5–10% in other batches. The actual number of cells involved in giant cell formation represented a small portion of the total hemocytes present in any particular monolayer preparation. Whereas a giant cell might contain >50 nuclei, it was unusual to find more than 5–10 giant cells per coverslip, and each preparation consisted of many thousands of hemocytes. Polykaryon formation was followed in 20 batches of clams collected at roughly monthly intervals, but its frequency did not correlate with any obvious parameters such as water temperature, photoperiod or size of organism. The clams were obtained from commercial sources, so nothing was known of their prior history, exposure to environmental stressors, etc.

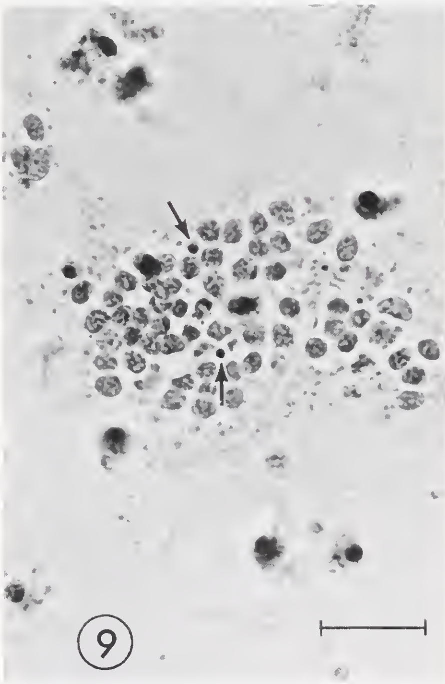
Attempts were made to augment giant cell formation *in vitro,* by the addition of foreign particles to the media overlying the hemocyte cultures, or *in vivo,* by injecting the particles directly into the adductor muscle hemolymph sinus, and taking hemolymph samples at intervals thereafter. The results of these studies will not be presented in detail because it was impossible to correlate statistically the frequency of giant cell formation with either of the treatments. Metal particles were phagocytosed *in vitro* to some extent, for example, carbon, graphite, aluminum, brass, or iron particles immediately adhered to the hemocytes and were ingested by 2–4 h. Silica and titanium dioxide were infrequently actually phagocytosed, but remained bound to hemocyte membranes. Considerable uptake of 2.02 $\mu$m diameter latex beads was seen, but phagocytosis of 1.10 $\mu$m diameter beads was minimal. Both FBC and LC were seen in many of the above-mentioned cultures, but at about the same frequency as in the untreated control preparations. Yeast particles were rapidly ingested *in vitro* and enclosed in large phagocytic vacuoles. In about half of the studies, phagocytic stimulation of the hemocytes by yeast cell exposure caused a marked increase in giant cell formation; however, repeated attempts to reproduce this phenomenon gave inconsistent results. The rate of giant cell formation *in vivo* by hemocytes taken from clams injected 1–48 h previously with particulates was comparable to that seen in controls. Hemocytes from these animals often had cell surface-bound and/or ingested particles. In some cases, *e.g.* 6 h after injection of 50 $\mu$g graphite in 0.5 ml SSW, the foreign material was recovered in clumps enveloped by cellular capsules.

If con A was present in the medium, the hemocytes underwent membrane alterations apparently leading to clumping and eventual fusion. The course of events following exposure to any given con A concentration was essentially the same, regardless of length of exposure (2–48 h). The typical appearance of an early (6 h) untreated hemocyte preparation is shown in Figure 11; the cells are rather evenly distributed over the glass substrate. However, if the cells from the same animal were exposed to Con A in the medium, the hemocytes showed a marked tendency to clump and form syncytial masses (Fig. 12).

Exposure of hemocytes to 60–250 $\mu$g/ml con A produced numerous FBC and LC, as well as cell aggregations, in the hemocyte preparations from >80% of the clams







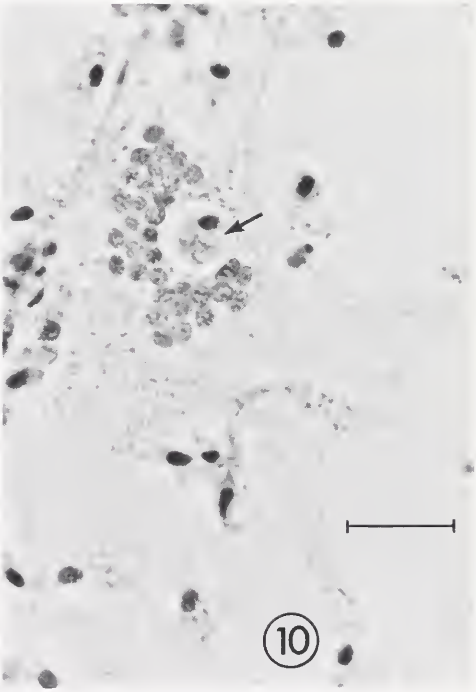
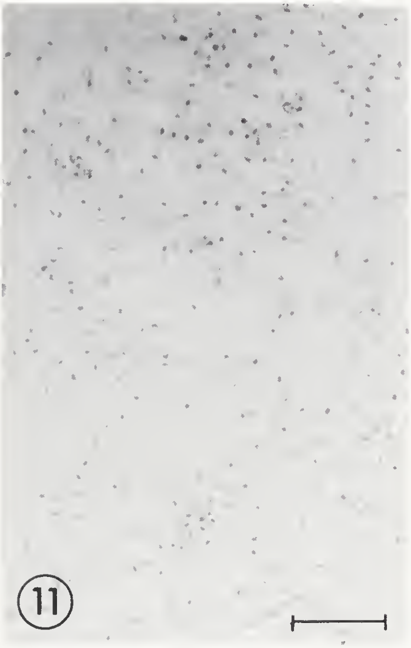


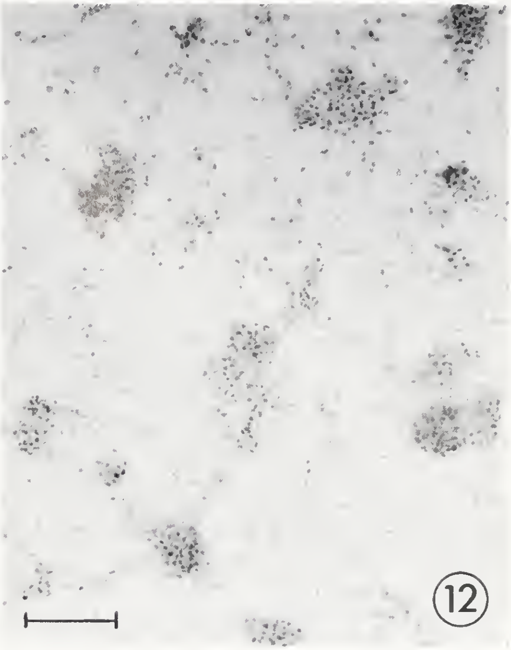
FIGURE 7. A mature LC in a 48 h preparation containing at least 80 nuclei.

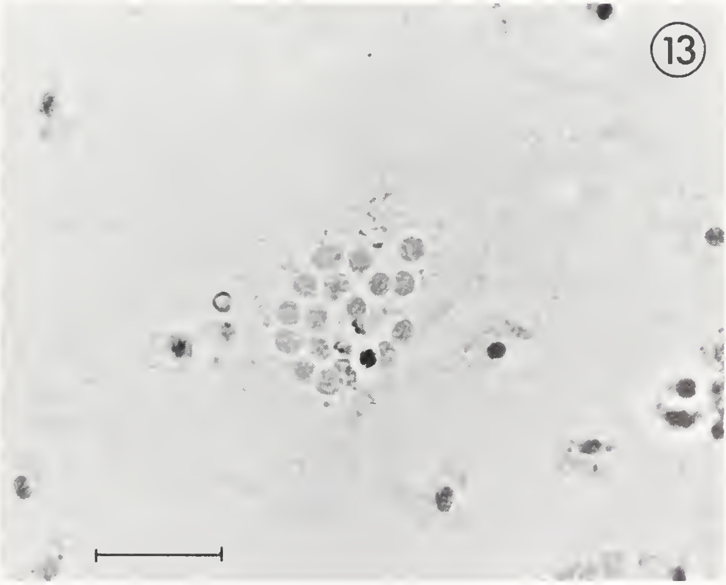
FIGURE 8. Two FBC (24 h) connected by cytoplasmic strands, the lower cell contains unidentified amorphous material (arrow).

FIGURE 9. A mature FBC (48 h) with randomly arranged nuclei and several yellow-brown inclusions (arrows).

FIGURE 10. A FBC (24 h) containing cell-like structure (arrow) in a large vacuole. Scale bar = 20 $\mu$m for Figures 7–10.







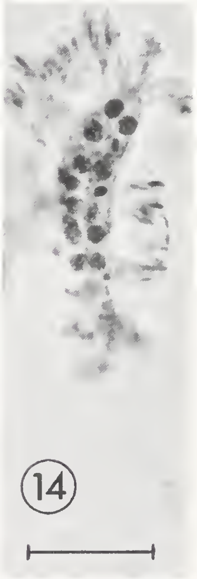


FIGURE 11. Untreated adherent hemocytes after 6 h; cells are randomly dispersed over the preparation. Scale bar = 200 $\mu$m.

FIGURE 12. Cells from sample hemolymph sample as in Figure 11. Adherent cells were exposed to 250 $\mu$g/ml con A for 1 h and maintained for 5 h in the absence of con A. Many foci of aggregation are present throughout the preparation. Scale bar = 200 $\mu$m.

FIGURE 13. Typical FBC in preparation exposed to con A (62.5 $\mu$g/ml, 24 h).

FIGURE 14. A FBC with highly ruffled membranes in a 24 h culture exposed to 125 $\mu$g/ml con A. Scale bar = 20 $\mu$m for Figures 13 and 14.

studied. Cellular adhesion to glass was increased by exposure to con A, as shown by the unusually dense cell pellets remaining after vigorous washing with SSW. The morphology of MGC produced in the presence of con A was generally similar to that in spontaneously produced polykaryons (Fig. 13); however, occasionally FBC with highly ruffled membranes were present (Fig. 14). At higher con A concentrations (>250 μg/ml), hemocyte clumping and disruption were pronounced. The cells appeared to be extensively lysed, and the preparations contained filamentous networks and flocculent masses containing entrapped nuclei and debris.

Careful examination of 500 hemocytes in each of 10 monolayer preparations showed no evidence of mitotic activity; similarly, no sign of nuclear division was seen in any of 20 randomly selected giant cells. The apparent mitotic figure in Figure 13 is an artifact which is not seen in other focal planes of this cell. Since mitosis is very rare in hemocytes, either immediately after withdrawal or during our *in vitro* studies, the role of endomitosis in *Mercenaria* giant cell formation is probably minimal.

## Discussion

It is now accepted that MGC in laboratory rodents and other vertebrates arise from fusion of mononuclear phagocytes, based on direct observation (Sutton and Weiss, 1966; Bayliss, 1976), incorporation of labeled macrophage nuclei into MGC (Mariano and Spector, 1974), and the production of hybrid enzymes in MGC exudates from chimeric mice composed of two strains, each homozygous for one of the two isoenzymic forms of glucose-6-phosphate isomerase (Murch *et al.*, 1982). The blood phagocytes of most invertebrates examined so far show a number of morphological and biochemical similarities to the mononuclear leukocytes of vertebrates (Anderson *et al.*, 1973; Anderson, 1975; 1977; 1981). The ability of *Mercenaria* hemocytes to form several classical types of MGC is taken as another example of the early phylogenetic origins of macrophage-like cells. Multinucleated giant cells seen in these clam hemocyte cultures were virtually identical to those described in vertebrates. Several lines of indirect evidence point to fusion as the means of MGC formation in *Mercenaria*. Mitotic figures were almost never seen in individual hemocytes or in polykaryons. Polykaryon formation was rare at low cell densities, when the individual hemocytes were not in contact with each other. Concanavalin A was shown to promote hemocyte aggregation and MGC formation.

It is not clear that FBC and LC are distinct cell types, many intermediate forms exist in both *Mercenaria* and in vertebrates. Early ideas that FBC were formed only in response to the presence of insoluble foreign substances and that LC were restricted to granulomata associated with certain infections have proven to be subject to numerous exceptions. Most evidence supports the view that LC and FBC are actually different states of the same cell (Roizman, 1962). While there are reports that LC are the precursors of FBC (van der Rhee *et al.*, 1978; 1979), it is generally thought that LC arise from FBC, following microtubule-dependent reordering of the nuclei and other organelles (Mariano and Spector, 1974; Adams, 1976; Chambers and Spector, 1982). The results of this study suggest that a similar transformation of FBC to LC may occur in *Mercenaria*, because LC usually appeared in culture later than FBC, and because the LC:FBC ratio generally increased between 12–48 h. Long-term study of MGC in *Mercenaria* was difficult because the cells apparently became nonadherent or lysed after several days in culture. Murine MGC were also reported to have a short lifetime (Papadimitriou and Sector, 1971), with the actual life span inversely proportional to the number of nuclei per cell (Papadimitriou and Walters, 1979).

The exact stimulus for MGC formation by *Mercenaria* hemocytes is unknown, but several suggestions have been proposed to account for macrophage fusion in higher animals. There are reports that fusion is directed by lymphokines released after antigenic stimulation (Galindo, 1972; Parks and Weiser, 1975), but many of the agents most commonly used to induce MGC formation are poorly antigenic. Indeed, thymectomy was shown to have little effect on MGC production (Papadimitriou, 1976). In this study, it is unlikely that soluble blood cell products, either secreted into the hemolymph or released from accidental hemocyte lysis during hemolymph collection and subsequent handling, mediated MGC formation. All hemocyte preparations were maintained in media containing pooled, cell-free hemolymph, which presumably would contain hemocyte products, but MGC formation was seen in only certain samples. The evidence strongly suggests that the capability to spontaneously form MGC was restricted to individual *Mercenaria,* and was not merely a result of *in vitro* culture of hemocytes. It was suggested that MGC were produced as a result of recognition of cell surface abnormalities on ageing or otherwise altered macrophages (Mariano and Spector, 1974), but various experimental alterations of macrophage surfaces failed to lead to the formation of polykaryons (Chambers, 1977a). Another possible theory (Chambers, 1977b) suggests that MGC form after the simultaneous attachment of several macrophages to the same ingestible material. The cells then fuse in the course of their efforts to ingest the same particle; cell membrane fusion normally accompanies the phagocytic process.

Attempts were made to promote MGC formation by *Mercenaria* hemocytes *in vivo* and *in vitro* by exposure to a variety of foreign particles. Whereas many kinds of particles were phagocytosed, in few cases were there clear increases in MGC formation caused by competition by the cells for the material in question, or as an incidental consequence of the cells ingesting material in close proximity to other hemocytes, as suggested by Chambers and Spector (1982).

The plant lectin con A promoted MGC formation in *Mercenaria* hemocyte cultures; both FBC and LC were more abundant after con A treatment. Berman and Stulberg (1962), Ptak *et al.* (1970), Smith and Goldman (1971), and Chambers (1977c, d) using lectins or antimacrophage serum, showed that if human or rodent macrophages are brought into contact before ingestion of surface-bound material, subsequent incubation will result in cell fusion. These molecules which induce MGC formation after interaction with the membrane may mimic the action of natural moieties generated in the inflammatory environment, and may account for the apparent lack of foreign material in certain MGC when examined under the microscope.

Irregular, yellow-brown granules, resembling those of brown or serous cells, were seen free in the cytoplasm of FBC, or still contained in apparently intact cells within the giant cells. It is interesting that cell-like inclusions can also be found in murine MGC (see Fig. 5 in Mariano and Spector, 1974). The central location of this material in clam FBC of all sizes suggests a possible role in MGC initiation, possibly following phagocytosis. The presence of foreign material in the cytoplasm suggested to early workers that augmented phagocytosis was a major function of MGC. However, more recent work showed that giant cells have a lower phagocytic potential than an equivalent mononuclear cell population. In fact, it was shown that both reduction in phagocytic activity and loss of cell surface receptors accompany the development of MGC (Papadimitriou *et al.,* 1975; Chambers, 1977e).

The granulomatous inflammatory response in vertebrate animals has received continuous scientific study for many years. Comparable information on invertebrates is not as comprehensive. Mammalian granulomas can be defined as a compact, organized collections of mature mononuclear phagocytes (Adams, 1976); frequently other elements of the mononuclear system are also present, including epithelioid cells and multinucleated giant cells. Giant cells are commonly associated with inflammatory responses to bacterial and viral infections, implanted foreign material, and/or neoplasms. Preliminary evidence presented here suggests that giant cells are also found in invertebrates, and that they are probably derived, as is the case in higher animals, from fusion of phagocytic leukocytes.

## ACKNOWLEDGMENTS

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# LOW TEMPERATURE EVOKES RAPID EXOCYTOSIS OF SYMBIOTIC ALGAE BY A SEA ANEMONE

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## ABSTRACT

Darkness evokes expulsion of zooxanthellae from the sea anemone *Aiptasia pulchella,* but brief exposure to low temperature (4°C, 4 h) increases the expulsion rate four-fold. Ninety-eight percent of the zooxanthellae are expelled within four days. Low temperature incubation had no detectable effect on host animal survival or behavior, but the effect on zooxanthellae was profound. Low temperature reduced the rate of photosynthesis, increased the rate of release of fixed carbon, reduced the number of viable cells and decreased the rate of cell division. Electron micrographs revealed that low temperature elicited the appearance of a thickened cell envelope and novel electron-dense inclusions, tentatively identified as crystallized lipoprotein. Immediately after low temperature incubation zooxanthellae move towards the apex of the host cells and are released to the coelenteron by exocytosis. The mechanisms by which low temperature may evoke exocytosis of zooxanthellae (*e.g.,* disassembly of host cell microtubules; increased concentration of cytosolic calcium ions) are discussed.

## INTRODUCTION

Symbiotic dinoflagellates (=zooxanthellae) are found in tropical reef cnidarians (*e.g.,* sea anemones, corals) in very high concentration relative to the surrounding water column. Under normal conditions neither the algae nor the host outgrows the other, suggesting that the algal population density is somehow regulated (Muscatine *et al.,* 1985). Yet, when environmental conditions on coral reefs are naturally perturbed (lowered salinity, abnormally high or low water temperature, sub-aerial exposure, and UV irradiation), zooxanthellae are lost from corals (Goreau, 1964; Jaap, 1979, 1985; Egana and Disalvo, 1982; Verrill, 1902, and Mayer, 1914, in Porter *et al.,* 1982; Glynn, 1983, 1984; Lasker *et al.,* 1984; Harriott, 1985; Fisk and Done, 1985). Additional types of stress conditions, brought to bear on symbiotic cnidarians by investigators in the laboratory or in the field, also evoke loss of zooxanthellae. These conditions include constant light, elevated temperature, elevated salinity (Reimer, 1971; see also Steele, 1976, 1977), prolonged darkness (Yonge and Nicholls, 1931; Franzisket, 1970; Kevin and Hudson, 1979), shading (Rogers, 1979), and anoxia (Yonge and Nicholls, 1931; O. Hoegh-Guldberg, pers. comm.).

Given the fundamental importance of zooxanthellae to reef corals and other symbiotic cnidarians (Muscatine, 1980), it is surprising that very little is known of the mechanisms by which zooxanthellae population densities are controlled, or how the various environmental stresses evoke elimination of zooxanthellae.

Steen (1985) recently noted that exposure of the tropical symbiotic anemone *Aip-*

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*tasia pulchella* to low temperature (4°C) for one hour in darkness, followed by maintenance at 25°C in darkness, resulted in rapid and substantial loss of zooxanthellae over the next few days. The host was otherwise unaffected. In view of the rapidity of the response and the ability of the host to survive, we have used low temperature treatment as a bioassay for the study of mechanisms by which zooxanthellae are eliminated from their host.

This paper describes aspects of the kinetics and cell biological features of the loss of zooxanthellae from a sea anemone in darkness and after brief exposure to low temperature. The results show that although darkness evokes loss of zooxanthellae, low temperature accelerates loss four-fold, increases the permeability of zooxanthellae to photosynthate, and alters zooxanthellae ultrastructure. The mechanism by which low temperature evokes rapid exocytosis of zooxanthellae is discussed.

## MATERIALS AND METHODS

### *Organisms and maintenance*

The tropical sea anemone *Aiptasia pulchella* (Clone B; Muller-Parker, 1984) was used for all experiments. Anemones were normally maintained in large finger bowls in 800 ml seawater in a Model 808 Precision Incubator at 25°C on a 12h:12h light: dark photoperiod (70 $\mu$Ein$\cdot$m$^{-2}\cdot$s$^{-1}$). Animals were fed freshly hatched *Artemia* nauplii twice weekly. After each feeding, seawater and uneaten *Artemia* were discarded and replaced with fresh seawater. When dark conditions were required, a similar incubator was used, without lights, and the temperature was adjusted to 25°C. Anemones were not fed during the experiments.

### *Counting numbers of zooxanthellae in sea anemones*

Samples of five anemones of similar size were homogenized in a glass-Teflon Potter homogenizer (10 ml), and the homogenate centrifuged in an IEC-HNS table centrifuge at 700 × *g* for 3 min to pellet the algae. The animal homogenate was decanted and the pellet preserved in seawater containing 4% formaldehyde. Numbers of algae per anemone were determined from hemacytometer counts of samples of formalin-fixed cells. Algal pellets were saved and used later for determination of mitotic index (see below).

### *Viability of zooxanthellae expelled from anemones*

Viability of zooxanthellae was assessed from levels of fixation of $^{14}$C and from observations on dye exclusion.

Expelled clumps of zooxanthellae were collected with a Pasteur pipette from bowls in which anemones were maintained. When sufficient cells were collected, the clumps were gently homogenized in several ml seawater and 5 replicate samples of 1 ml each were placed in separate 12 ml conical graduated centrifuge tubes. Samples were taken for cell counts. Then, 0.1 ml of a stock solution of $NaH^{14}CO_3$ containing one $\mu$Ci per ml was added to each tube. After mixing the contents of each tube, 25 $\mu$l of the stock solution was immediately taken for estimation of Added Activity. Tubes were incubated for 2 h at 300 $\mu$Ein$\cdot$m$^{2}\cdot$s$^{-1}$ at 25°C and mixed again after one h. After incubation, tubes were centrifuged at 1000 × *g* for 4 min to pellet the algae, and the labelled seawater supernatant was decanted and saved. The algae were resuspended in 0.2 ml distilled water, the suspension was transferred to glass scintillation vials, acidified with 0.1 ml 1 *N* HCl, and evaporated to dryness with low heat on a warming plate. Samples of labelled supernatant were treated similarly. Then 10 ml scintillation cocktail was added to each vial. For Added Activity, samples were added to 10 ml of scintillation cocktail, and the vials were capped immediately. Vials were counted in a Beckman LSC Model 100 scintillation counter, and corrected for quench by the external standards ratio method. Results are expressed as $\mu g$ C fixed $\cdot 10^6$ cells$^{-1} \cdot h^{-1}$.

For the dye exclusion assay, expelled zooxanthellae were collected, and placed on a standard microscope slide. A drop of 0.25% (w/v) Evans Blue (Gaff and Okong'o-Ogola 1971) was added and the percentage of cells permeated by the dye after 5 min was established from observation of five replicate samples of 200 cells at 450×.

### *Mitotic index*

Mitotic index was determined for zooxanthellae in the same samples used to measure zooxanthellae population density. The number of cells undergoing cytokinesis was determined for 10 samples of 100 cells, each viewed at 450× on a hemacytometer grid to facilitate counting.

### *Specific growth rates and loss rates of zooxanthellae*

Specific growth rates of zooxanthellae *in situ* were calculated from

$$\mu = 1/N\,(N/t)$$

where N = the standing stock of zooxanthellae and (N/t) the increment of new cells added per day (Strickland and Parsons, 1965). By analogy, specific expulsion rates were calculated from

$$\mu_x = 1/N\,(N_x/t)$$

where $N_x$ is the number of algae expelled, calculated from N at $t_0 - t + 10$ days, and $N_x/t$, the average increment of cells expelled per day (O. Hoegh-Guldberg, pers. comm.).

### *Morphology and ultrastructure*

For transmission electron microscopy whole anemones and discharged pellets of algae were fixed in 4% formaldehyde and 1% glutaraldehyde in 0.1 *M* phosphate buffer according to McDowell (1978). Tentacle crowns were removed and subdivided into small pieces, rinsed in buffer, and dehydrated in a series of ethanol solutions (30%, 50%, 70%, 90%, and three changes of absolute ethanol), 30 min at each step. Tissues were transferred to absolute ethanol:propylene oxide (1:1) for 1 h, then into propylene oxide:medcast epoxy (1:1) and finally into 100% medcast epoxy for infiltration overnight. Tissues were embedded in fresh 100% medcast and the material was hardened according to the manufacturer's instructions.

Thick sections of tentacles and pellets were cut with glass knives, stained with methylene blue and mounted on standard microscope slides. These were viewed and in some cases photographed with a Olympus BH-2 compound microscope using Panchromatic BW film (ASA 200). Some tentacles were deliberately cut in cross section to facilitate determination of the intracellular locus of individual algae with respect to the long axis of their host endodermal cell. This locus is expressed in terms of $R_f$, the ratio of the distance from (a) the base of the endoderm cell to the leading edge of the alga, to (b) the base of the endoderm cell to the center of the tentacle lumen. The latter criterion was used because the apex of each endoderm cell usually could not be discerned.

Thin sections of tentacles or algal pellets were cut with diamond or glass knives, collected on 200 mesh copper grids coated with parlodion and carbon film, and stained with uranyl acetate and lead citrate. Grids were examined with a Philips 300 Electron microscope operating at 60 kV.

For scanning electron microscopy, excised tentacles and discharged pellets were fixed in 4% formalin in seawater. Tentacles were slit longitudinally with a scalpel blade and all specimens were critical-point dried in a Polaraon Critical-Point Drier, mounted on stubs, sputter-coated with gold, and viewed with an ISI-DS-130 scanning electron microscope.

## RESULTS

### *The effect of darkness on maintenance of zooxanthellae population density*

Since our bioassay was carried out in cold and darkness, it was necessary to quantify and thereby control for loss of algae from *A. pulchella* due to darkness alone. Groups of *A. pulchella* that were starved for one day were incubated for 4 h in darkness at 25°C. Thereafter they were maintained in darkness without feeding for up to 20 days. Five anemones were sampled at zero time (immediately after treatment) and at two-day intervals, and the number of zooxanthellae remaining in each anemone was determined. The results are shown in Figure 1. In these anemones, the numbers of algae decreased steadily, and over ten days the standing stock of algae decreased by 58%. ($\mu_x = 0.06\ d^{-1}$). Clumped pellets of zooxanthellae were found in the experimental containers in the vicinity of individual anemones during the experiment, indicating that the loss of substantial numbers of zooxanthellae was due to expulsion from the anemone. Digestion of some algae could have occurred as well, but this possibility was not investigated.

### *Effect of a brief exposure to low temperature on the maintenance of zooxanthellae population density*

Anemones incubated at 14°C for 4 h in darkness and then maintained at 25°C in darkness lost zooxanthellae at a faster rate ($\mu_x = .08\ d^{-1}$) than controls, losing about 72% of their algae in 10 days (Fig. 1). But when anemones were incubated at 4°C in darkness and then 25°C in darkness, loss of zooxanthellae was even more rapid. After only 4 days the population of algae had decreased by 98% ($\mu_x = .24\ d^{-1}$). Further decrease in numbers was only slight over the next 20 days. The combination of darkness and low temperature incubation caused relatively rapid loss of roughly 98–99% of the algae associated with *A. pulchella.*

Low temperature incubation had no obvious visible effects on the host anemone behavior. They exhibited normal posture (column erect, tentacles extended) and feeding behavior, and continued to produce buds by pedal laceration for at least six months after low temperature treatment. However, in one case, several tentacle tips, 1–2 mm long and filled with zooxanthellae, were recovered from incubation dishes. Presumably these had sloughed or broken off during or after low temperature treatment.

### *Effect of light on loss of zooxanthellae after low temperature incubation*

To determine if the loss of zooxanthellae after low temperature treatment in darkness was affected by light, a group of anemones was incubated at 4°C in darkness as usual, but then maintained in the light (70 $\mu\text{Ein}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) for 10 days. Anemones were sampled at two-day intervals and numbers of zooxanthellae per anemone deter-

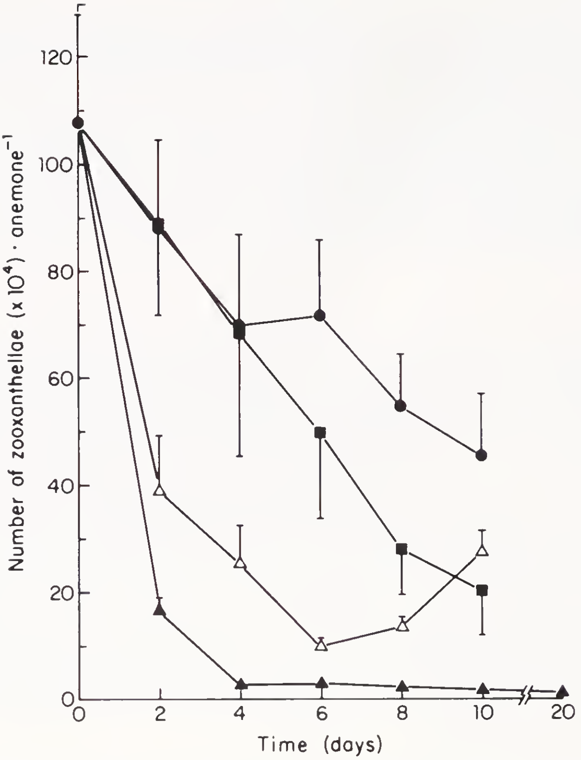


FIGURE 1. Number of zooxanthellae remaining in *Aiptasia pulchella vs.* time in dark at 25°C after an initial 4-h incubation at 25°C (●), 14°C (■), and 4°C (▲); and time in light at 25°C after an initial 4-h incubation in dark at 4°C (△). Vertical bars indicate standard error of the mean.

mined. Figure 1 shows that such individuals lost abundant zooxanthellae immediately and at approximately the same rate as those that were chilled and maintained in darkness, but the rate of loss decreased slightly after 2 days until, by day 6, about 91% of the original population had been expelled. Then, over the next 4 days, the population began to increase at a specific growth rate of about 0.44 $d^{-1}$.

### *Viability of cold-treated zooxanthellae*

To determine if low temperature incubation affected the viability of zooxanthellae, we tested the ability of freshly isolated and expelled zooxanthellae to photosynthetically fix $^{14}C$ and to exclude dye. The results are given in Table I.

Zooxanthellae freshly isolated (FIZ) from control anemones (25°C, dark) fixed $^{14}C$ at normal rates (*cf.* Muller-Parker, 1984), and about 98–99% of these zooxanthellae were viable as judged from ability to exclude dye. FIZ from cold (4°C) treated anemones assayed immediately or after a 4-h recovery period also fixed $^{14}C$ but at much lower rates. Of these cells, 91.8–93.6% were judged viable. Extending the recovery period at 25°C to 24 h resulted in a modest increase in photosynthetic rates, but the rates were still below control levels and viability was reduced to 82.5%.

FIZ customarily release a fraction of their fixed $^{14}C$ to the external medium (Muscatine, 1980). Those from *A. pulchella* (Clone B) normally release less than 10% of

TABLE I

*Photosynthetic rate ($\bar{x} \pm$ S.D.) and % viability (dye exclusion) of zooxanthellae freshly isolated (FIZ) and/or expelled (EZ) from control (25°C dark) and chilled (4°C dark)* Aiptasia pulchella

| Host treatment | Recovery period before assay | Cells assayed | Photosynthetic rate ($\mu gC \cdot 10^6$ cells$^{-1} \cdot h^{-1}$) | Viable cells (%) | Cell-specific photosynthetic rate ($\mu gC \cdot 10^6$ viable cells$^{-1} \cdot h^{-1}$) |
|---|---|---|---|---|---|
| 25°C dark | | | | | |
| 4 h | — | FIZ | 9.63 ± 1.57 | 98.7 | 9.76 |
| | | | 4.15 ± 0.53 | 98.5 | 4.22 |
| | | | | | $\bar{x}$ = 6.99 ± 3.92 |
| 4°C dark | | | | | |
| 4 h | — | FIZ | 0.51 ± 0.05 | 91.8 | 0.56 |
| 4 h | 25°C 4 h, dark | FIZ | 0.70 ± 0.09 | 93.6 | 0.74 |
| 4 h | 25°C 24 h, dark | FIZ | 2.89 ± 0.21 | 82.4 | 3.50 |
| | | EZ | 1.53 ± 0.13 | 82.3 | 1.86 |
| | | | | | $\bar{x}$ = 1.42 ± 1.02 |

the total $^{14}C$ fixed in a one-hour incubation (O. Hoegh-Guldberg, pers. comm.). Our controls released 28.8% under similar conditions (n = 5). In contrast, zooxanthellae from cold-treated anemones released an average of 58.8 ± 3.4% (n = 5) of the total $^{14}C$ fixed. Therefore, the cold treatment seemed to reduce rates of photosynthesis, increase the percentage of fixed $^{14}C$ released, and reduce numbers of viable cells.

Expelled zooxanthellae (EZ) were similar to FIZ with respect to $^{14}C$ fixation. Algae expelled up to four hours after cold treatment showed greatly reduced rates of photosynthesis and about 61% viability. Those expelled during a recovery period of 4–24 hours at 25°C showed some increase in rate of photosynthesis and level of cell viability, but the mean cell-specific rate of photosynthesis (1.42 ± 1.02 $\mu gC \cdot 10^6$ cells$^{-1} \cdot h^{-1}$) was still significantly lower than that of freshly isolated controls (6.99 ± 3.92; $P < 0.05$, Student's *t*-test). Release of fixed $^{14}C$ by EZ was not assayed.

### *Effect of low temperature on cell division*

The mean MI for zooxanthellae from *A. pulchella* (Clone B) maintained normally is 0.76% (Wilkerson *et al.*, 1983), and ranges from 0.38 to 1.54% (Muller-Parker, 1984). Figure 2 shows that mitotic index of zooxanthellae from controls incubated at 25°C ranged from 1.65% at time zero to 4.6–5.1% after 2–4 days in darkness and then declined again to 2% after 10 days. In contrast, algal cells from anemones incubated at 14° or 4°C had lower mitotic indices, ranging between 1.25 and 0.25%. These results indicate that darkness gave rise to a transient increase in MI and that brief exposure to low temperature may inhibit such an increase. Zooxanthellae from anemones incubated in light at 25°C after low temperature incubation in darkness exhibit a higher mitotic index, suggesting that light may reverse the inhibitory effect of low temperature on cell division.

### *Morphological correlates of expulsion of zooxanthellae*

*Intracellular loci of zooxanthellae.* Zooxanthellae are confined to the endoderm cells of *A. pulchella*. Most endoderm cells contain 1–3 algae, each within an animal cell vacuole (Glider *et al.*, 1980). To gain insight into morphological correlates of

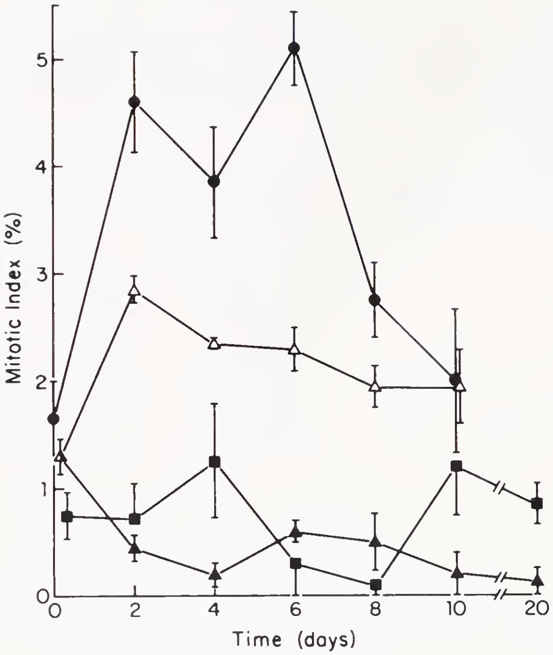


FIGURE 2. Mitotic index (%) of zooxanthellae in *Aiptasia pulchella vs.* time in dark (or light) at 25°C after initial treatment as in Figure 1. Symbols and vertical bars as in Figure 1.

expulsion, the loci of algae along the long axis of host endoderm cells, before and after exposure to darkness and low temperature, were determined from $R_f$ measurements of 80–200 cells in thick sections of tentacles. Results are shown in Figure 3. Zooxanthellae in cells of controls maintained in darkness for 4 h at 25°C were evenly distributed along the long axis of host cells. During prolonged maintenance in darkness, the population shifted toward the apical end of the host cells. In cold-treated anemones, this shift was detectable even at time zero. Within 24 hours after the cold treatment, the pattern appears reversed, but it is likely that the zooxanthellae near the luminal face of the host cell had already been expelled. The apical shift is restored after two days and considerably exaggerated after four days. These data are consistent with the interpretation that a change in intracellular locus precedes and accompanies the discharge of zooxanthellae.

*Ultrastructure of zooxanthellae* in situ *and during expulsion.* Zooxanthellae *in situ* in anemones maintained in light at 25°C are shown in Figure 4a. The cell envelope consists of two to five sets of double membranes between the presumptive animal cell vacuolar and zooxanthellae plasma membranes. The average thickness of the envelope is about 60 nm. The chloroplast is lobed, with a prominent chloroplast membrane and lamellae in the three-thylakoid configuration. The pyrenoid (Fig. 4a, inset) is congruent with the chloroplast stroma but apparently not traversed by thylakoids. It is capped by a thick sheath, presumably consisting mainly of starch. The nucleus contains condensed chromosomes. Mitochondria are present and adjacent to the pyrenoid there is a large amorphous body and crystal-like electron translucent profiles.

Zooxanthellae *in situ* in the anemones incubated at 4°C and sacrificed at day 0

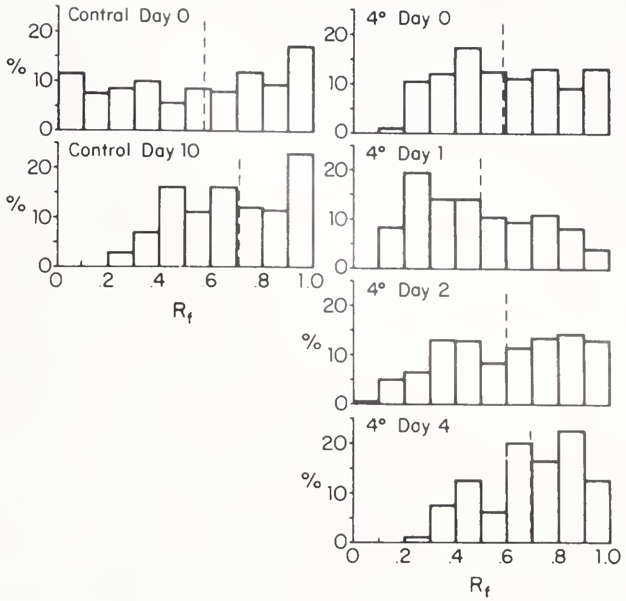
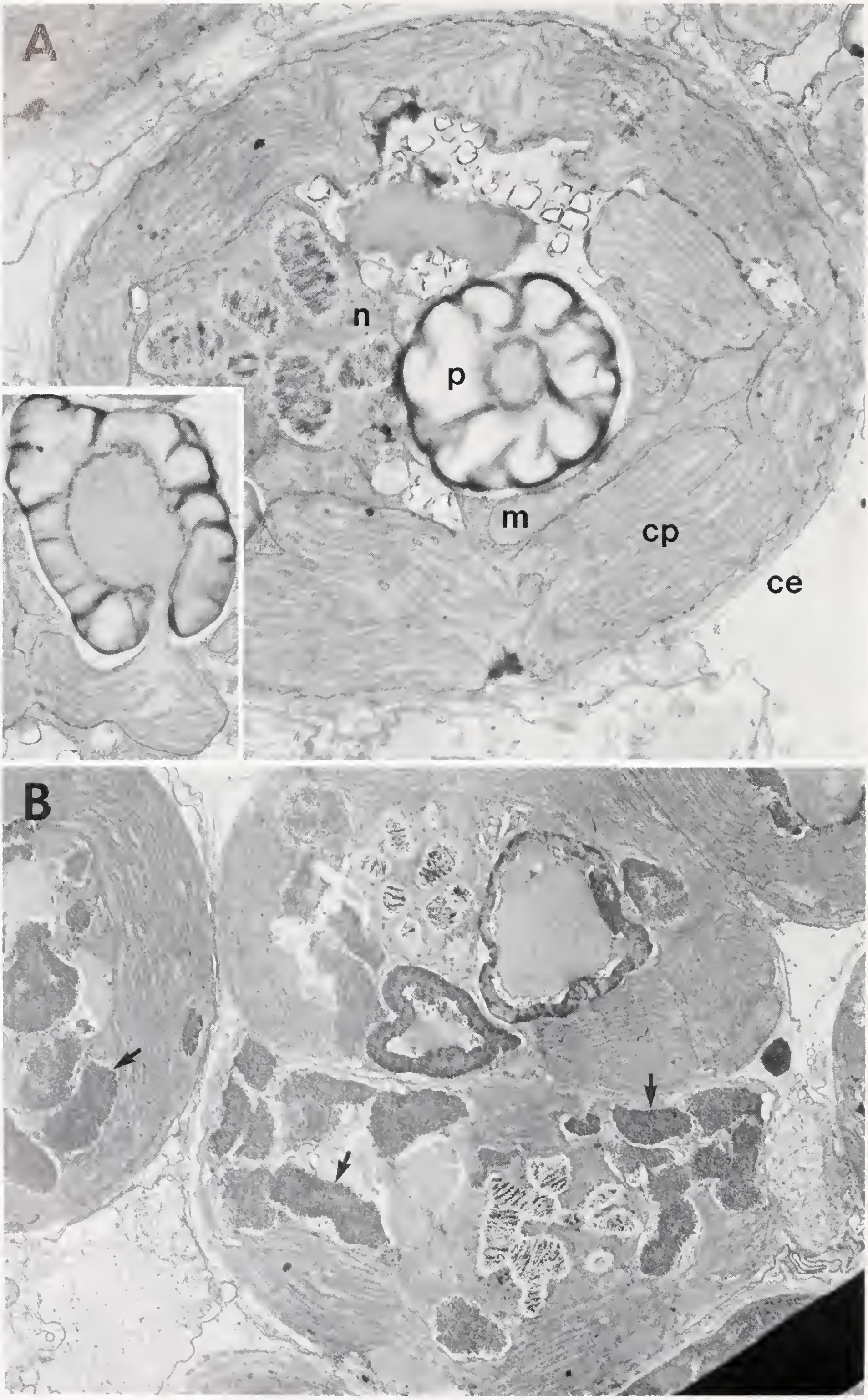


FIGURE 3. Number of zooxanthellae (%) *vs.* relative position along the long axis of host cells in control (25°C, dark 4 h) and chilled (4°C, dark 4 h) anemones. Dashed line indicates mean $R_f$.

(immediately after cold treatment) are shown in Figure 4b. In some cells the average thickness of the cell envelope has increased and there is loss of resolution of cell envelope membranes. The most striking change is manifested by the appearance of electron-dense inclusions and an increase in the density of the pyrenoid starch cap. After one day (Fig. 5a), cell envelope thickness has increased to about 120 nm. The dense inclusions are less granular and now exhibit a dense core. Dense core inclusions are also present in the host cell. Electron-dense inclusions are still evident in zooxanthellae in anemones sacrificed after two days (not shown).

*Release of zooxanthellae from tentacle cells.* The inner surface of tentacles is lined with flagellated endoderm cells, each containing zooxanthellae. In tentacles of control anemones, the zooxanthellae are situated proximally within the host cells and the endodermal epithelium is relatively smooth (not shown). As a result of chilling, the zooxanthellae move distally within the host cells, causing the distal end of the host cell to bulge into the tentacle lumen and to assume the spherical shape of the zooxanthellae they contain. Low power scanning electron micrographs of the tentacle endoderm immediately after chilling give the general appearance of a tentacle lined with extracellular zooxanthellae (Fig. 6a). However, at this time most of the zooxanthellae are still within the host cells. Figure 6b shows the flagella at the apex of the extended host cells and in one case a torn host cell shows that it persists as a thin enclosure for a zooxanthellae cell. About 8 h after chilling, zooxanthellae can be seen emerging from host cells, apparently by exocytosis (Fig. 6c). By 12 h the terminal stages of exocytosis and release of zooxanthellae can be observed (Fig. 6d). By 20 h most of the zooxanthellae have been discharged from the epithelium. Those few that remain are also in late stages of exocytosis (Fig. 7a).

Algae are discharged from the coelenteron as individual cells or as pellets of cells. The pellets are irregularly spherical, and also contain bacteria and assorted fibrils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and sheets of material of unknown origin and composition (Fig. 7b). Transmission electron micrographs of pellets (Fig. 5b) show that some zooxanthellae are apparently intact and exhibit a thick cell envelope, while others are in various stages of disintegration. Each is surrounded by one or more thin membranes raised above the surface of the cell. Figure 7c shows that the algae are generally spherical but enclosed by a loose wrinkled sheath probably corresponding to the elevated membranes shown in Fig. 5b). In cells *in situ* this sheath is torn apart and shed as the two daughter cells undergo cytokinesis (Fig. 7d) and is therefore interpreted as a mother cell envelope.

## Discussion

### *The effect of darkness on maintenance of zooxanthellae population density*

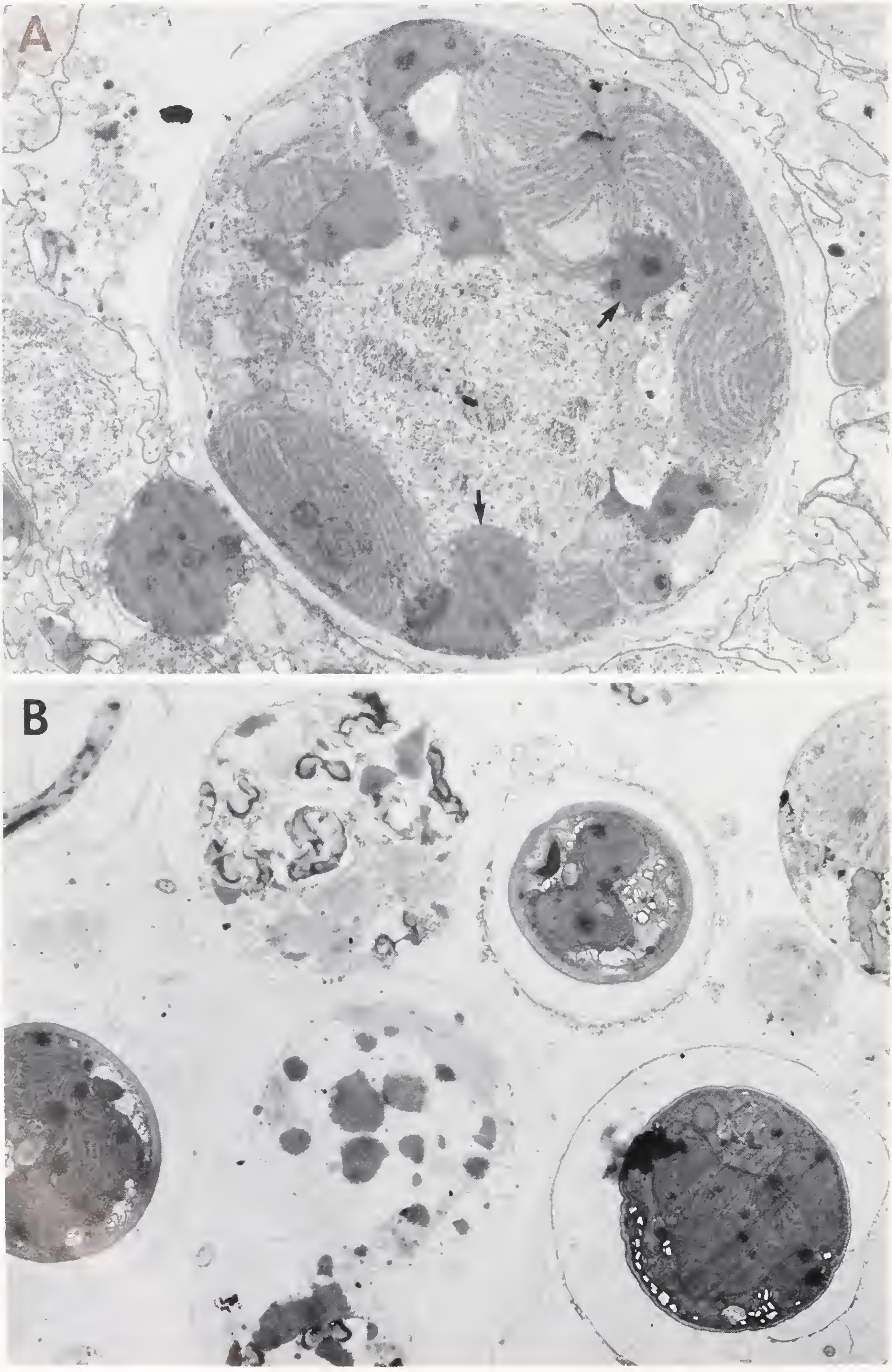
The results of this investigation show that when the symbiotic anemone *Aiptasia pulchella* (Clone B) is maintained in darkness, without feeding, it progressively loses zooxanthellae. These results confirm the observations of Muller-Parker (1984), that darkness causes loss of zooxanthellae from *A. pulchella* (Clone B), and extend them by showing that at least 50% of the zooxanthellae are lost in 10 days.

Previous studies have demonstrated that symbiotic anemones and corals lose zooxanthellae in prolonged darkness (Yonge and Nicholls, 1931; Buchsbaum, 1968; Franzisket, 1970; Kevin and Hudson, 1979; Rogers, 1979; Muller-Parker, 1984), but neither the time course nor the mechanism of loss has been rigorously investigated. More recently, Glider (1983) demonstrated that symbiotic *Aiptasia pallida* maintained on a 12 h light:12 h dark photoperiod continuously extrude pellets of zooxanthellae but in significantly greater numbers during the dark period.

In the case of *A. pulchella,* loss of zooxanthellae in darkness could occur by expulsion or digestion. The appearance of numerous clumps of zooxanthellae in the vicinity of individual anemones shows that loss is due, at least in part, to expulsion. Data from Figure 3 show that algae are initially distributed more or less evenly along the long axis of the host cell. In darkness, they move toward the distal end of the host cell and are then released from host cells by exocytosis (Fig. 6c, d). In the coelenteron they form large pellets which are then egested. Egestion of regularly formed pellets of zooxanthellae by *A. pallida* and by Hawaiian zoanthids has been described by Reimer (1971). The mechanism which evokes intracellular migration and exocytosis of zooxanthellae in darkness is unknown but may be correlated with diminished algal photosynthesis. In this connection, we note that the maintenance of symbiotic *Chlorella* sp. in the cells of green hydra may depend on the sustained translocation of photosynthetically fixed carbon from the algae to the host cells (Hohman *et al.*, 1982).

There is evidence for presumed digestion of zooxanthellae in some symbiotic cnidarians (Fitt and Trench, 1983) but the possibility was not investigated in this study. The remainder of this discussion will address loss of zooxanthellae by expulsion only.

FIGURE 4. (a) Transmission electron micrograph of a zooxanthella in *Aiptasia pulchella* maintained at 25°C on a 12L:12D photoperiod. ce, multilayered cell envelope; cp, chloroplast; m, mitochondrion n, nucleus with condensed chromosomes; p, pyrenoid, 20,383×. Inset, chloroplast-pyrenoid complex, 17,976×. (b) Zooxanthella from an anemone maintained 4 h at 4°C in dark. Electron-dense inclusions at arrows. 11,194×.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### *The effect of low temperature on maintenance of zooxanthellae population density*

Brief exposure to low temperature increased the rate of loss of zooxanthellae slightly at 14°C but four-fold at 4°C (Fig. 1). We speculate that low temperature could potentiate exocytosis by increasing the rate of microtubule depolymerization (Melkonian *et al.,* 1980; Dustin, 1984). For example, in green hydra, endoderm cells form endocytic vacuoles upon uptake of symbiotic *Chlorella* sp. These vacuoles migrate away from the site of uptake (Muscatine *et al.,* 1975). Migration is thought to be mediated by microtubules since the movement of vacuoles containing algae is inhibited by colchicine (Cook, unpub., in Muscatine *et al.,* 1975) or vinblastine (McAuley and Smith, 1982). Treatment of green hydra with vinblastine, which promotes microtubule disassembly, causes the algae, which are normally situated at the base of the host cell, to gradually shift back toward the apex of the cell (L. Muscatine, unpub.). Low temperature could also stimulate exocytosis by causing an increase in cytosolic calcium ions (Campbell, 1983). Increased cytosolic calcium has been implicated in a wide range of systems involving stimulus-secretion coupling (See, for example, Douglas, 1973; Dahl and Henquin, 1978; Spearman and Butcher, 1982).

### *Effect of light on loss of zooxanthellae after low temperature incubation*

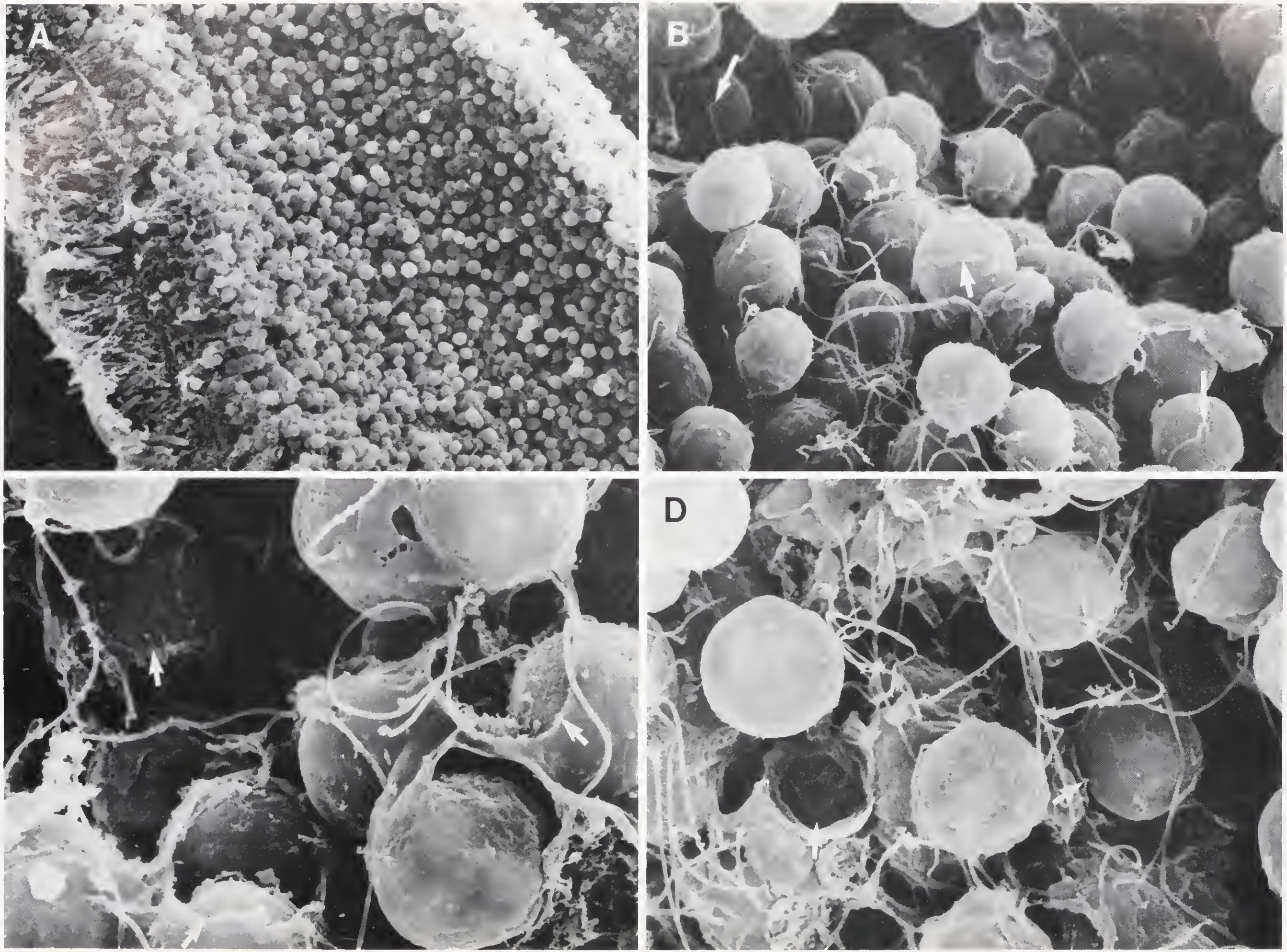
Maintenance of *A. pulchella* in light after low temperature incubation in darkness did not significantly affect loss of zooxanthellae for up to six days. This suggests that the effect of low temperature incubation is not quickly or easily reversed by the restoration of photosynthesis or translocation, or the putative reassembly of microtubules. After six days, the standing stock of algae begins to increase, suggesting that the effect of low temperature is no longer manifested.

### *Viability of cold-treated zooxanthellae*

Zooxanthellae expelled by *A. pallida* are photosynthetically active (Glider, 1983), as are zooxanthellae freshly isolated from *A. pulchella* after incubation for 4 h in darkness at 25°C (Table I). However, incubation of *A. pulchella* at 4°C caused a decrease in photosynthetic rate and impaired the ability of freshly isolated or expelled cells to exclude dye. This effect of low temperature was only partially reversible at 24 h, again suggesting that metabolism was substantially altered. This conjecture is supported by data showing that release of fixed $^{14}$C by chilled cells was higher than release by controls. Table I also shows that, since the percentage of viable cells in samples of expelled zooxanthellae was less than the percentage in freshly isolated zooxanthellae, the cells impaired by chilling may be preferentially expelled.

Interestingly, the viability of zooxanthellae freshly isolated from controls was 98.6%. These cells were separated from the host by homogenization and centrifugation. However, if cells are isolated by excising a tentacle and gently pressing it with a glass rod, then 100% of the cells are able to exclude the Evans Blue dye. This implies that either a small proportion of the zooxanthellae are damaged by the homogenization method or that non-viable cells are derived from other parts of the anemone.

FIGURE 5. (a) Transmission electron micrograph of a zooxanthella from an anemone incubated 4 h in dark at 4°C, then 24 h in dark at 25°C. Electron-dense inclusions at arrows. 18,846×. (b) Transmission electron micrograph of a pellet of zooxanthellae discharged after 5 days from anemones treated as in 5a. 7780×.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### *Effect of low temperature on cell division*

The MI of zooxanthellae increased from the normal range of 0.38% to 1.54% to 4.6% to 5.1% after several days in darkness. The reason for this increase is not yet known, but a similar result was obtained by Glider (1983). He found that MI was greater in darkness in zooxanthellae both *in situ* in *A. pallida* and in expelled pellets. Given that algae are expelled in darkness, the effect of increased division rate in darkness is to offset the rate of expulsion. Since low temperature decreases MI (Fig. 2) it is possible that accelerated loss arises merely from a reduction in the rate of replacement. Calculations addressing this possibility (Appendix 1) suggest that it is highly unlikely that accelerated loss arises solely from inhibition of division (replacement) rate.

### *Morphological correlates of expulsion of zooxanthellae*

Transmission electron micrographs of chilled zooxanthellae *in situ* show a thickened cell envelope. The significance of the thickened envelope is not yet understood but it does resemble the "pellicle" which develops when zooxanthellae are grown in culture (Schoenberg and Trench, 1980; Glider, 1983). The nature of the electron-dense entities in zooxanthellae cytoplasm which do not appear in controls is unknown. Puppione *et al.* (1982) suggest that triglyceride-rich lipoprotein enriched in saturated fats, upon exposure to temperatures sufficiently low to induce phase transition, forms crystallized particles enclosed within a phospholipid monolayer. If zooxanthellae contain such materials in membranes, and if they crystallize at low temperature, membrane function might be perturbed. Such a perturbation might explain the observed increased release of photosynthate by chilled cells. In this connection, Harnischfeger and Jarry (1982) report that cooling *Chlorella emersonii* to around −5°C causes a reversible alteration in thylakoid fluidity which in turn increases the permeability of thylakoids to cations.

Glider (1983) observed transmission electron micrographs of host cells during normal expulsion of zooxanthellae. Algae at the distal end of host cells were covered with a thin layer of cytoplasm connected to the cell by a narrow tissue bridge. This profile was interpreted as imminent "budding" rather than classical exocytosis. The animal tissue covering was thought to be digested in the coelenteron since it was not present in zooxanthellae pellets. We suggest that Glider (1983) observed the apical "bulging" of host cells caused by the extreme distal migration of zooxanthellae. While it is possible that some zooxanthellae are released by "budding," our scanning electron micrographs (Figs. 6b–d) offer direct evidence to support the interpretation that zooxanthellae are discharged by exocytosis. Further, the loose, wrinkled sheath which we observed in pellet zooxanthellae (Fig. 7c) more likely corresponds to a mother cell envelope, since it seems to be intimately associated with the cells during cytokinesis.

Studies now in progress show that brief exposure to low temperature evokes exocytosis of zooxanthellae in *A. pallida* from Bermuda and *Aiptasia* sp. from Java, but not in several scleractinian corals from Bermuda. The specificity of this phenomenon is now under investigation.

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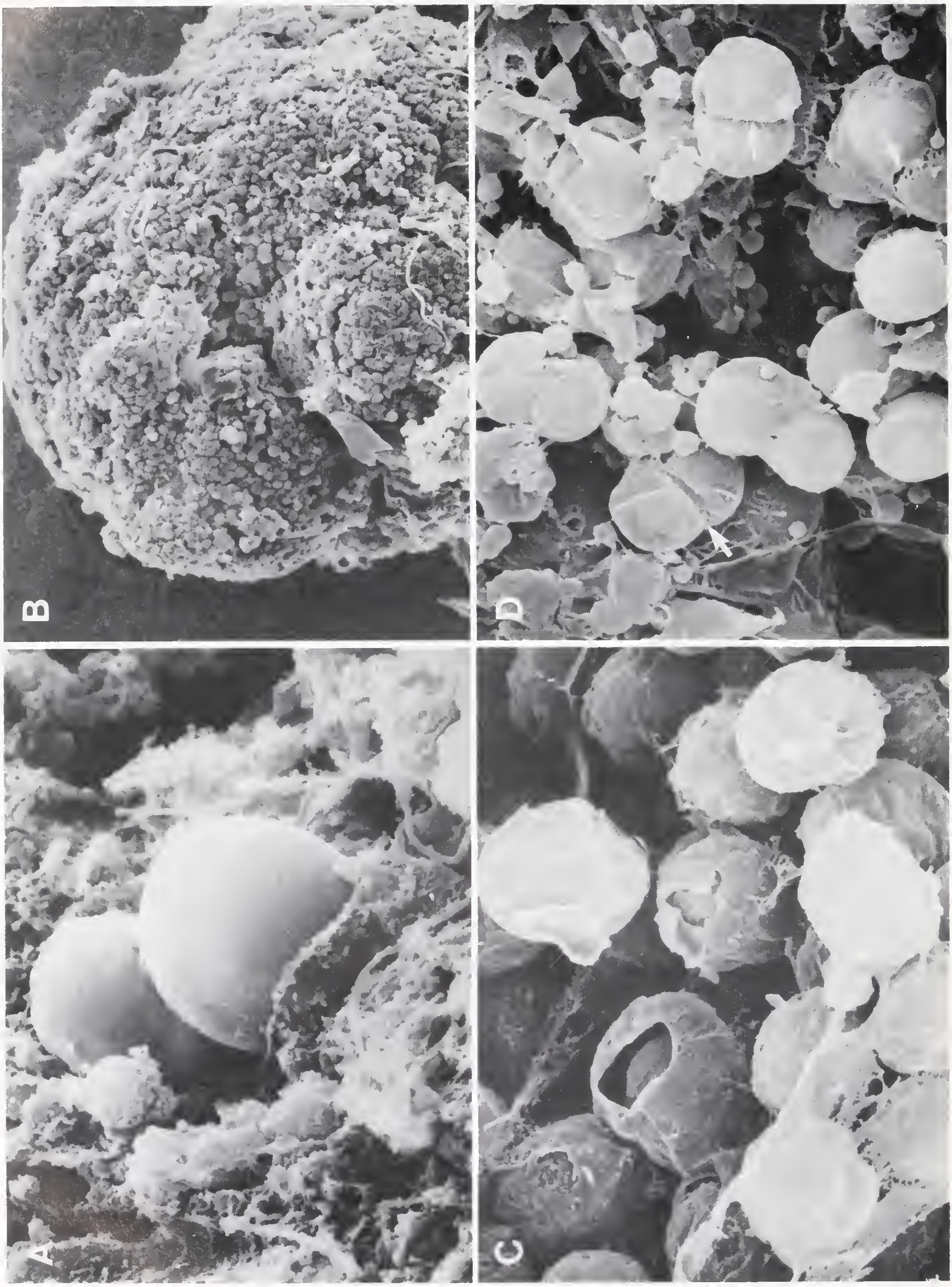
FIGURE 6. (a) Inner surface of *Aiptasia pulchella* tentacle immediately after chilling, showing endoderm and abundance of zooxanthellae. 234×. (b) As above, showing that zooxanthellae are still within host cells. Host cell flagella and torn host cell revealing zooxanthella surface at arrows. 2400×. (c) As above, 8 h after chilling, showing zooxanthellae within and emerging from host cells (at arrows). 4750×. (d) As above, 12 h after chilling, showing zooxanthella freed from exocytotic cup (arrows). 3250×.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## APPENDIX 1

Let $N_t$ = standing stock of algae per anemone at time t

$$\frac{dN_t}{dt} = \begin{array}{c}\text{number of algae produced}\\ \text{per unit time}\end{array} - \begin{array}{c}\text{number of algae expelled}\\ \text{per unit time}\end{array}$$

Let m = number of algae produced per alga per unit time, and

let x = probability of an algae being expelled per unit time.

Then,

$$\frac{dN_t}{dt} = N_t(m - x)$$

and since

$$\frac{dN_t}{dt} \cdot \frac{1}{N_t} = \mu, \qquad \text{then}$$

$$\mu = m - x$$

Our task is to compute

$$\mu_{25} = m_{25} - x_{25}$$

and $\mu_4 = m_4 - x_4$, and to compare $x_{25}$ and $x_4$.

From Figure 1 we know $N_t$, so we can calculate $\mu$ at 25° and 4°C from

$$N_t = N_0e^{\mu t}$$

This yields

$$\mu_{25} = -.08, \text{ and } \mu_4 = -.92$$

Then,

$$m = 1/t_d \ln(1 + f),$$

where $t_d$ = duration of cytokinesis and f = the average daily mitotic index. Assuming that $t_d$ is the same at 25° and 4°C, and taking the value of $t_d = 0.46$ days (Wilkerson *et al.*, 1983), then from Figure 2

$$f_{25} = 3.33\% \text{ and } f_4 = 0.54\%,$$

and

$$m_{25} = 0.71 \text{ and } m_4 = 0.12$$

FIGURE 7. (a) Endodermal surface of tentacle of *Aiptasia pulchella*, 20 h after chilling, showing a pair of zooxanthellae in late stage of exocytosis from host cell, and paucity of zooxanthellae elsewhere. 5910×. (b) Pellet of zooxanthellae discharged by chilled anemone. 241×. (c) Zooxanthellae in discharged pellet, each with a wrinkled envelope and connected by mucus strands. 3590×. (d) Zooxanthellae in tentacle lumen of anemone treated 4 h, 4°C in dark, showing various aspects of zooxanthellae mother cell envelope during cytokinesis. 2430×.

Therefore,

$$\begin{aligned} x_{25} &= m_{25} - \mu_{25} \\ &= .07 - (-.08) \\ &= .15 \end{aligned}$$

and

$$\begin{aligned} x_4 &= .01 - (-.92) \\ &= .93 \end{aligned}$$

Therefore the probability of a zooxanthella being expelled after chilling is six times higher than that at 25°C.

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# THE BIOLOGICAL BULLETIN

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# INSTRUCTIONS TO AUTHORS

*The Biological Bulletin* accepts outstanding original research reports of general interest to biologists throughout the world. Papers are usually of intermediate length (10–40 manuscript pages). Very short papers (less than 10 manuscript pages including tables, figures, and bibliography) will be published in a separate section entitled "Short Reports." A limited number of solicited review papers may be accepted after formal review. A paper will usually appear within four months after its acceptance.

The Editorial Board requests that manuscripts conform to the requirements set below; those manuscripts which do not conform will be returned to authors for correction before review.

1. ***Manuscripts.*** Manuscripts, including figures, should be submitted in triplicate. (Xerox copies of photographs are not acceptable for review purposes.) The original manuscript must be typed in double spacing (*including* figure legends, footnotes, bibliography, etc.) on one side of 16- or 20-lb. bond paper, 8½ by 11 inches. Manuscripts should be proofread carefully and errors corrected legibly in black ink. Pages should be numbered consecutively. Margins on all sides should be at least 1 inch (2.5 cm). Manuscripts should conform to the *Council of Biology Editors Style Manual*, 4th Edition (Council of Biology Editors, 1978) and to American spelling. Unusual abbreviations should be kept to a minimum and should be spelled out on first reference as well as defined in a footnote on the title page. Manuscripts should be divided into the following components: Title page, Abstract (of no more than 200 words), Introduction, Materials and Methods, Results, Discussion, Acknowledgments, Literature Cited, Tables, and Figure Legends. In addition, authors should supply a list of words and phrases under which the article should be indexed.

2. ***Figures.*** Figures should be no larger than 8½ by 11 inches. The dimensions of the printed page, 5 by 7¾ inches, should be kept in mind in preparing figures for publication. We recommend that figures be about 1½ times the linear dimensions of the final printing desired, and that the ratio of the largest to the smallest letter or number and of the thickest to the thinnest line not exceed 1:1.5. Explanatory matter generally should be included in legends, although axes should always be identified on the illustration itself. Figures should be prepared for reproduction as either line cuts or halftones. Figures to be reproduced as line cuts should be unmounted glossy photographic reproductions or drawn in black ink on white paper, good-quality tracing cloth or plastic, or blue-lined coordinate paper. Those to be reproduced as halftones should be mounted on board, with both designating numbers or letters and scale bars affixed directly to the figures. All figures should be numbered in consecutive order, with no distinction between text and plate figures. The author's name and an arrow indicating orientation should appear on the reverse side of all figures.

3. ***Tables, footnotes, figure legends, etc.*** Authors should follow the style in a recent issue of *The Biological Bulletin* in preparing table headings, figure legends, and the like. Because of the high cost of setting tabular material in type, authors are asked to limit such material as much as possible. Tables, with their headings and footnotes, should be typed on separate sheets, numbered with consecutive Roman numerals, and placed after the Literature Cited. Figure legends should contain enough information to make the figure intelligible separate from the text. Legends should be typed double spaced, with consecutive Arabic numbers, on a separate sheet at the end of the paper. Footnotes should be limited to authors' current addresses, acknowledgments or contribution numbers, and explanation of unusual abbreviations. All such footnotes should appear on the title page. Footnotes are not normally permitted in the body of the text.

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A. Journal abbreviations, and book titles, all underlined (for *italics*)

B. All components of abbreviations with initial capitals (not as European usage in World List *e.g. J. Cell. Comp. Physiol.* NOT *J. cell. comp. Physiol.*)

C. *All* abbreviated components must be followed by a period, whole word components *must not* (*i.e. J. Cancer Res.*)

D. Space between all components (*e.g. J. Cell. Comp. Physiol.*, not *J.Cell.Comp.Physiol.*)

E. Unusual words in journal titles should be spelled out in full, rather than employing new abbreviations invented by the author. For example, use *Rit Vísindafjélags Islendinga* without abbreviation.

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G. The order of abbreviated components should be the same as the word order of the complete title (*i.e. Proc.* and *Trans.* placed where they appear, not transposed as in some Biological Abstracts listings).

H. A few well-known international journals in their preferred forms rather than World List or USASI usage (*e.g. Nature, Science, Evolution* NOT *Nature, Lond., Science, N.Y.; Evolution, Lancaster, Pa.*)

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# MARINE BIOLOGICAL LABORATORY CALENDAR OF CENTENNIAL EVENTS

The Marine Biological Laboratory was founded in 1888. The centennial of this founding will be recognized by the laboratory through a series of special events. This series will include special lectures, seminars, symposia, and workshops. The centennial celebration will begin in the summer of 1987 and extend through 1988.

These events should encompass many fields of biology and provide "state-of-the-art" knowledge about them. Collectively, they should be of general interest to biologists. Therefore, a cumulative calendar of these events will be published in *The Biological Bulletin* beginning with this issue.

—The Editor

## GAMETE DIALOGUE IN FERTILIZATION: FROM SEA URCHIN TO HUMAN*

A MARINE BIOLOGICAL LABORATORY CENTENARY SYMPOSIUM IN HONOR OF ALBERTO MONROY

*23–25 August 1987*
*Marine Biological Laboratory*
*Woods Hole, Massachusetts 02543*

### Sunday, August 23

12 noon–7 pm: Registration. Informal Reception 5–7 pm.

### Monday, August 24

8:45–9:00 am: Opening remarks by C. B. Metz (U. Miami).

9:00–12:00 noon: Session I: *Responses of sperm to the egg's environment.* G. S. Kopf, Chairperson.

*Speakers:* G. S. Kopf (U. Pennsylvania)
M. Hoshi (Tokyo Institute of Technology)
D. L. Garbers (Vanderbilt University)
D. P. Wolf (Oregon Regional Primate Research Center)
S. Meizel (U. California, Davis)

1:30–4:30 pm: Session II: *Reactions comprising sperm/egg recognition and binding.* B. Shapiro, Chairperson.

*Speakers:* B. Shapiro (U. Washington)
R. De Santis (Stazione Zoologica, Napoli)
E. Carroll (U. California, Riverside)
B. Storey (U. Pennsylvania)
N. Cross (U. California, Davis)

---

* Program subject to change.

**Tuesday, August 25**

9:00–12 noon: Session III: *Reactions comprising sperm/egg plasma membrane fusion.* D. Myles, Chairperson.

*Speakers:* D. Myles (U. Connecticut)
T. Chambers/D. McCulloh (U. Miami)
J. Hedrick (U. California, Davis)
R. Harrison (AFRC Cambridge)
P. Saling (Duke University)

1:30–4:30 pm: Session IV: *Egg responses to sperm/egg fusion.* G. Schatten, Chairperson.

*Speakers:* G. Schatten (U. Wisconsin)
L. Jaffe (U. Connecticut)
M. Whitaker (U. London)
R. M. Schultz (U. Pennsylvania)
G. Guidice (U. Palermo)

4:30–5:00 pm: Summation of meeting by D. Epel (Hopkins Marine Station).

5:00–5:15 pm: Closing remarks by L. Mastroianni (U. Pennsylvania).

*The Alberto Monroy memorial symposium is sponsored by the National Institute of Child Health and Human Development, The Rockefeller Foundation, and the Marine Biological Laboratory. The symposium has been coordinated by Luigi Mastroianni, Jr., Chair; Jerry L. Hedrick; Gregory S. Kopf; and Bayard T. Storey.*

For further information please write:

Ms. Debbie Coffin
Division of Reproductive Biology
Medical Labs 311
Department of Obstetrics and Gynecology
University of Pennsylvania
Philadelphia, PA 19104-6080

## THE SECOND SYMPOSIUM ON REGULATORY ACTIONS OF GROWTH FACTORS IN REPRODUCTION AND EARLY DEVELOPMENT

A SYMPOSIUM HELD IN CELEBRATION OF THE CENTENNIALS OF THE NATIONAL INSTITUTES OF HEALTH (1887–1987) AND THE MARINE BIOLOGICAL LABORATORY (1888–1988)

*1–2 September 1987*
*Marine Biological Laboratory*
*Woods Hole, Massachusetts 02543*

Participants in the Symposium will review and assess the current state of knowledge of the molecular mechanisms of the regulation of reproductive and early developmental events. The Symposium will focus on the interactions and interrelationships of growth factors, reproductive hormones, and their receptors in the regulation of cell function and responsiveness to environmental signals. Autocrine and paracrine modes of growth factor action in the reproductive system or during early development of the embryo will be discussed. In addition, consideration will be given to the extent and possible significance of extensive homologous sequences in the structures of relevant growth factors, oncogenes, reproductive hormones, and receptors.

Interested scientists are invited to submit a 200-word abstract of work to be presented. The deadline for abstract submission is

**1 July 1987**

*The Symposium is sponsored by the Reproductive Sciences Branch, Center for Population Research, National Institute of Child Health and Human Development.*

For further information, please call or write to:

Dr. William A. Sadler
Chief, Reproductive Sciences Branch
National Institute of Child Health and Human Development
7910 Woodmont Avenue, Room 7C33
Bethesda, Maryland 20892
Telephone: (301) 496-6515

## OPTICAL APPROACHES TO THE DYNAMICS OF CELLULAR MOTILITY

A Marine Biological Laboratory Centenary Symposium in Honor of Robert D. Allen

*5–8 October 1987*
*Marine Biological Laboratory*
*Woods Hole, Massachusetts 02543*

### Monday, October 5

8:00 pm: Wine and cheese party and demonstrations of optical equipment by manufacturers.

### Tuesday, October 6

Morning: Introductory remarks by N. Kamiya (Okazaki)

Morning session: *Optical techniques in cell biology.* D. L. Taylor and S. Inoué, Chairpersons.

*Speakers:* G. Nomarski (Antony, France)
S. Inoué (Marine Biological Laboratory)
D. L. Taylor (Carnegie Mellon)
D. A. Agard (U. California Medical School, San Francisco)
T. Yanagida (U. Osaka)

Afternoon session: *Amoeboid movement and cytoplasmic streaming.* J. Condeelis, Chairperson.

*Speakers:* N. Kamiya (Okazaki)
J. Condeelis (Albert Einstein)
M. Schliwa (U. California, Berkeley)
J. Hartwig (Mass. General, Harvard University)
G. Oster (U. California, Berkeley)

Evening session: Talks and demonstrations by equipment manfuacturers (Swope Center).

*Speakers:* R. Wicks (PMI Corp.)

*Speakers* (*cont'd*): Zeiss representative
Remainder of program to be arranged

### Wednesday, October 7

Morning session: *Mitosis.* L. I. Rebhun, Chairperson.

*Speakers:* Y. Hiramoto (Tokyo)
E. W. Salmon (U. North Carolina, Chapel Hill)
A. Bajer (U. Oregon, Eugene)
L. I. Rebhun/R. Palazzo (U. Virginia)
W. C. Cande (U. California, Berkeley)

Afternoon session: Posters.

Evening session: *Motility in other systems.* K. Edds, Chairperson.

*Speakers:* N. S. Allen (Wake Forest)
R. Hard (SUNY, Buffalo)
J. Hayden (Sienna College)
J. Lafountain (SUNY, Buffalo)
C. Izzard (SUNY, Albany)

### Thursday, October 8

Morning session: *Microtubule dynamics.* R. Sloboda, Chairperson.

*Speakers:* R. Sloboda/S. Gilbert (Dartmouth College)
J. Travis (Vassar College)/S. Bowser (NY State Dept. of Health, Albany)
T. Tchen (Wayne State)
P. Satir (Albert Einstein)
H. Hotani (Kyoto University)

Afternoon session: *Axoplasmic transport.* D. Weiss, Chairperson.

*Speakers:* D. Weiss (Munich)/G. Langford (U. North Carolina, Chapel Hill)
R. Smith (Edmonton, Canada)
A. Breuer (Cleveland Clinic)
B. Schnapp (NIH/Marine Biological Laboratory)
R. Weisenberg (Temple University)

Evening: Dinner and concert.

*Abstracts* may be submitted for inclusion in the poster session and will be published in *Cell Motility and Cytoskeleton.* Information on preparation of abstracts will be sent with application forms for registration. Final date for abstract submission will be **1 August 1987.**

*Registration and Housing:* The registration fee is $75. Housing is limited and will be assigned in order of receipt of application. Deadline for housing and meal plan is **1 August 1987.** Forms and information may be obtained by writing:

Robert Day Allen Symposium
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# THE EVOLUTION OF THE VERTEBRATE PLASMA PROTEINS*

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## ABSTRACT

The appearance of vertebrate animals some 450 million years ago was heralded by the invention of a number of new proteins that are found in the blood plasma, including albumin and other transport proteins, the non-enzyme blood clotting and complement proteins, and a host of protease inhibitors. Comparisons of present day amino acid sequences from various species allow us to look back to how and when many of these proteins originated.

## INTRODUCTION

It is particularly gratifying for me to present this Friday Evening Lecture on the evolution of vertebrate plasma proteins, since it is a project I began many years ago here at the MBL. At the time I was a graduate student at Harvard in a laboratory devoted to the study of human blood proteins, and, after having spent a summer in Woods Hole working in the area of comparative physiology, it was only natural that I should consider a comparative study of blood proteins from other vertebrates. The question immediately presented itself as to where, in the biological world, the characteristic proteins of blood are found, and from this gradually emerged the more profound questions as to when and how they evolved.

A typical mammalian blood plasma—the fluid in which blood cells are suspended—contains upwards of 600 protein components, as can be shown by high resolution two-dimensional electrophoresis (Anderson *et al.,* 1984). Some of these materials are doubtless only altered forms of particular proteins that result from various processing events; still, the multitude of individual types is impressive. In mammals, a single protein, albumin, accounts for half the protein mass of the plasma, however, and five more—haptoglobin, fibrinogen, transferrin, $\alpha$-1-antitrypsin and $\alpha$-2-macroglobulin—make up another quarter; lipoproteins and immunoglobulins constitute another large fraction (Table I). This evening I am going to concentrate on the origins of some of these most abundant proteins, although I will touch upon a few of the less abundant ones in passing.

Regarding the phyletic distribution of the plasma proteins, we can state flatly that many of these proteins, but certainly not all of them, are found in the blood plasmas of all the major classes of vertebrates, from fish to mammals. It has been alleged by some that albumin is absent from the blood of fish, although some workers have reported that it is present in assorted teleosts in small amounts (for a review, see Doolittle, 1984). There is general agreement that amphibians, reptiles, and birds all have albumins that are similar to the mammalian type. Only a few of the vertebrate plasma proteins have counterparts among the invertebrates, and, as we shall see, albu-

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TABLE I

*Most abundant proteins found in mammalian blood plasma*

| Protein | Abundance* (gm/liter) | Internal duplications | Carbohydrate |
|---|---|---|---|
| Albumin | 45 | Yes | No |
| Immunoglobulins | 15 | Yes | Yes |
| Lipoproteins | 10 | Yes | Yes |
| Haptoglobin | 6 | Yes | Yes |
| Fibrinogen | 3.5 | Yes | Yes |
| Transferrin | 3 | Yes | Yes |
| $\alpha_1$-Antitrypsin | 3 | No | Yes |
| $\alpha_2$-Macroglobulin | 2.5 | No | Yes |
| | 87.5 | | |

* These eight (sets of) proteins account for more than 95% of the mass of plasma proteins in mammals. The values given are approximate and vary somewhat from species to species and among individual species.

min is not among them. We'll return to the matter of occurrence shortly, but there are a few definitional matters to be settled first.

## FUNCTIONS OF THE PLASMA PROTEINS

The plasma proteins can be grouped functionally. Quite apart from albumin, there are numerous transport proteins, including transferrin (iron transport) and the lipoproteins. Indeed, any potential metabolite that is poorly soluble in plasma is likely to have a corresponding protein for rendering it soluble so that it can be transported from one place to another.

In addition to the transport proteins, the plasma contains numerous polypeptide hormones and growth factors, many in precursor forms awaiting proteolytic activation upon the appropriate signal. There are also numerous defense proteins, including the immunoglobulins and the associated complement system, and there is a set of proteins whose function is to prevent loss of the blood: the coagulation proteins. The latter is a special interest of mine, and I will be giving it an undue amount of attention this evening.

The complement and blood coagulation systems, and some other schemes in the plasma, depend upon a complex plan of proteolytic activation for their normal operation. Indeed, much of the extracellular way of life in multicellular creatures is regulated by a judicious and limited proteolysis. Whereas intracellular regulation depends largely on ATP-dependent phosphorylation (kinases) and subsequent hydrolysis (phosphatases), extracellular regulation derives its energy potential at the time of protein biosynthesis. It is then "leaked out" at some later time by a gradual and successive proteolysis.

As a consequence, blood plasma is rich with proteases and protease precursors. There are very many related serine proteases involved, and some sulfhydryl proteases. In order to keep the system stable, there are large amounts of general and specific protease inhibitors. Two of these, $\alpha$-2-macroglobulin and $\alpha$-1-antitrypsin, appear on our list of the most abundant plasma proteins (Table I), and they will figure heavily in our evolutionary discussion.

## HOW NEW PROTEINS ARE FORMED

At this point let me make some sweeping statements about the evolution of proteins in general. First, most new proteins evolve from old proteins as a result of gene

TABLE II

*Divergence times of some major vertebrate groupings**

| Vertebrate groupings | Millions of years ago |
|---|---|
| Old World monkeys/humans | 28 ± 2 |
| Artiodactyls/primates | 80 ± 10 |
| Primates/rodents | 80 ± 10 |
| Birds/mammals | 200 ± 20 |
| Reptiles/mammals | 200 ± 20 |
| Amphibia/mammals | 320 ± 30 |
| Bony fish/mammals | 375 ± 35 |
| Cyclostomes/mammals | 450 ± 50 |

* Groupings are based on the fossil record.

duplication. DNA has a propensity for duplicating itself under all sorts of conditions, and segments of all sizes are constantly being randomly and tandemly repeated. There are many known mechanisms for this excessive internal duplication, including unequal and homologous crossing over and other genetic delinquencies, but the important thing for the present discussion is that these duplications are observed in all living forms. If the region of duplicated DNA is short and inside the boundaries of the gene for a particular protein, then the protein is lengthened by the process, some portion of it now showing up twice or more. If the DNA duplication encompasses the entire gene—starts and stops included—then for a brief moment two genes will exist where there used to be one, and two separate products may be issued. Only one of these need be subject to natural selection, of course, and the other will be free to change, mostly as a result of errant base substitutions leading to single amino acid replacements. Usually such duplicons are destined for the genomic scrap-pile, since sooner or later a mutation will occur that will decommission the whole enterprise, but occasionally some new function—with benefit to the organism—will be encountered by chance. And with that, a *bona fide* "new" protein is eligible for the registry. It is related to the "old protein" of course, and depending on the rate of amino acid replacement along their divergent courses, the two will, for a long time, be recognizable merely on the basis of their amino acid sequences alone. By "a long time" I am implying time frames of a 100 million to several billion years (Table II).

## SETTING MOLECULAR CLOCKS

There are two independent means at our disposal for finding when a "new protein" made its appearance during evolution. We have already alluded to one of these, which simply amounts to surveying the biological community to see which creatures have the protein in question. If all mammals have some protein—*e.g.*, lactalbumin, which is found in milk—and none of the other classes of vertebrates have this protein, then we can presume that lactalbumin was "invented" some time around or just before the appearance of mammals, about 200 million years ago. If all vertebrates except fish have some particular protein, then we would expect that protein must have evolved about 375 million years ago, since that is when fish and the other vertebrates last shared a common ancestor (Table II).

The second way to gauge when a gene duplication leading to a new protein occurred, involves the comparison of amino acid sequences and the use of the "molecular clock." The method can also be used to find when an internal duplication leading

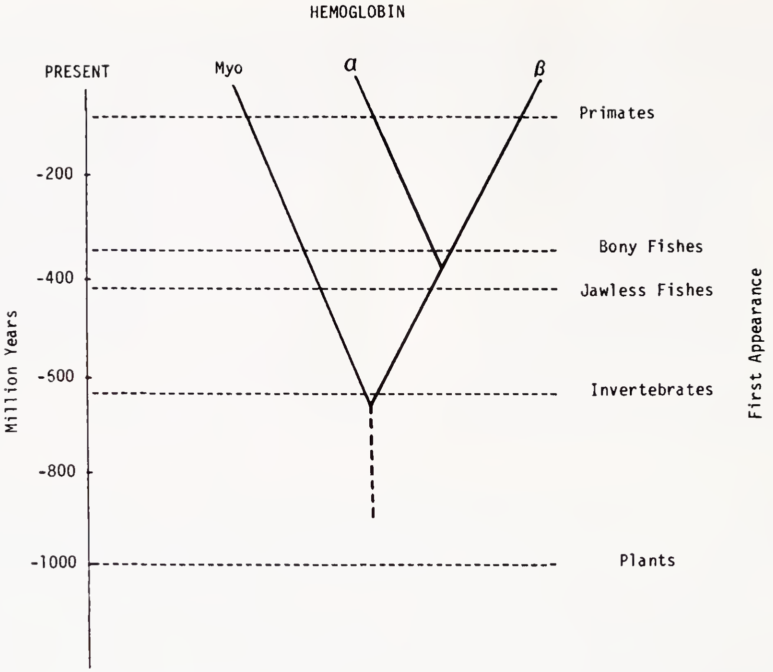

FIGURE 1. Gene duplication event that led to the existence of hemoglobin α and β chains. The timescale is based on how fast the amino acid sequences are changing as determined by species comparisons (from Doolittle, 1984).

to an internal repeat may have taken place. Although this is a very useful method, it is often subject to a certain amount of misinterpretation, and it is worth our while to remind ourselves how it works. First, it is a fact that the sequences of various proteins change at different but characteristic rates. For many proteins, as we shall see, the rate of change is really quite constant. In some situations, nonetheless, the rate of change of a particular type of protein may speed up or slow down, and we must be on the watch for a certain amount of quirkiness in molecular clocks. When they run smoothly they'll be in accord with what we find by the "occurrence method."

Consider a well known example. The α and β chains of vertebrate hemoglobin have been sequenced from many different species in all five classes. By consulting the divergence times in Table II, which are based on the fossil record, and quantitatively comparing the sequences from members of each group, we can estimate that the two chains are each changing at a rate of about 11 amino acid replacements per 100 residues per 100 million years. Today the two sequences in most vertebrates are 55% different (45% identical). As it happens, sequence change follows an exponential course (because of back mutations and multiple changes at the same site), and a 55% difference is actually equivalent to 90 actual changes for every 100 residues. At a rate of 11 per 100 million years, then, the time since the duplication that gave rise to divergence must be about 400 million years (keep in mind both proteins are changing). That being the case, we can make a prediction, assuming the clock has been ticking accurately. Any vertebrates that diverged from the mainline longer than 400 million years ago ought not to have the benefit of that duplication (Fig. 1). Indeed, cyclostomes (lampreys and hagfish) branched off about 450 million years ago, and those creatures have single-chained hemoglobins: no β chains!

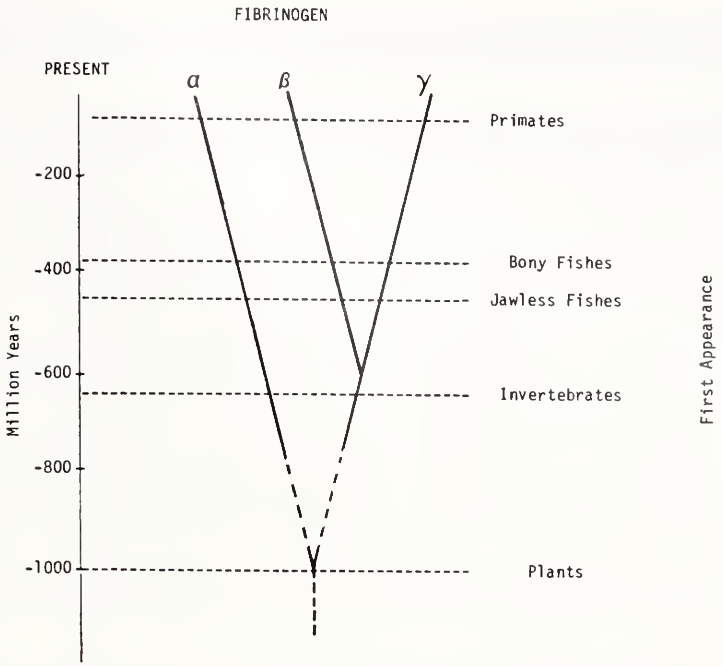

FIGURE 2. Gene duplication events that lead to three different polypeptide chains in vertebrate fibrinogens. See also Figure 1 (from Doolittle. 1984).

## FIBRINOGEN

I began my studies on the evolution of the plasma proteins with a consideration of the proteins involved in blood coagulation, a phenomenon which in mammals involves the interplay of a dozen different protein factors and that culminates in the conversion of the soluble protein fibrinogen into the insoluble gel fibrin. How could this system evolve? Of what utility would any portion of the cascade be without the remainder? These were the original questions, and I must admit at once that they remain mostly unanswered in a strict sense, although at this point a number of feasible scenarios can be drawn.

Blood clotting follows a similar pattern in all vertebrates, from the cyclostomes to the mammals. Lampreys have a fibrinogen molecule that is fundamentally the same as the human kind. It has three polypeptide chains ($\alpha$, $\beta$, and $\gamma$), and it is clotted by the proteolytic enzyme thrombin, which in lampreys is also similar to its human counterpart (Doolittle *et al.,* 1962). During the 1970's the complete amino acid sequence of human fibrinogen was unraveled, and the results confirmed earlier speculation that the three polypeptide chains were descended from a common ancestral type (Fig. 2). Thus, the $\beta$ and $\gamma$ chains are about 35% identical, and both of the latter are recognizably similar to parts of the $\alpha$ chain, although the resemblance is somewhat lower in the latter cases. Apparently the duplication leading to the divergence of the $\alpha$ and non-$\alpha$ chains occurred much longer ago than the one leading to $\beta$ and $\gamma$ chains.

Recently we completed the sequences of the $\beta$ and $\gamma$ chains of lamprey fibrinogen; in each case the sequences are just about 50% identical with the corresponding human sequence (Figs. 3, 4). That these two independent gene products have experienced such similar amounts of change proves that the molecular clock in this particular

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                  .  .  .. ....... .. .. .   .. .       . .   .   .
Human     YVATRDNccILDERFGSYcPTTcGIADFLSTYQTKVDKDLQSLEDILHQVENKTSEVKQL
Lamprey    QVRDLKQcSNDPEFGRYcPTTcGVADVLSKYAKGVDEDSSFIDSVLTQLAAKHGIVEGN

                             .       . ...    .     ..  .  . .    . .
Human     IKAIQLTYNPDESSKPNMIDAATLKSRKMLEEIMKYEASILTHDSSIRYLQEIYNSNNQK
Lamprey   VNIVNEDVRITRDEAQIIKDSGQKTVQKILEEVRILEQIGVSHDAQIQELSEMWRVNQQF

             .          .  .. ..    .  .......     . . .... ...... . .
Human     IVNLKEKVAQLEAQcQEPcKDTV  QIHDITGKDcQDIANKGAKQSGLYFIKPLKANQQF
Lamprey   VTRLQQQLVDIRQTcSRPcQDTTANKISPITGKDcQQVVDNGGKDSGLYYIKPLKAKQPF

          .. ...   ...... . . .... .   .  . .... . .. .............
Human     LVYcEIDGSGNGWTVFQKRLDGSVDFKKNWIQYKEGFGHLSPTGTTEFWLGNEKIHLIST
Lamprey   LVFcEI ENGNGWTVIQHRHDGSVNFTRDWVSYREGFGYLAPTLTTEFWLGNEKIHLLTG

          . .  . ..  . ..      ...  ..  .. . ... .     .... ..........
Human     QSAIPYALRVELEDWNGRTSTADYAMFKVGPEADKYRLTYAYFAGGDAGDAFDGFDFGDD
Lamprey   QQA  YRLRIDLTDWENTHRYADYGHFKLTPESDEYRLFYSMYLDGDAGNAFDGFDFGDD

          . ... . . .. ...   .... .. ............ ........ .. .. . .
Human     PSDKFFTSHNGMQFSTWDNDNDKFEGNcAEQDGSGWWMNKcHAGHLNGVYYQGGTYSKAS
Lamprey   PQDKFYTTHLGMLFSTPERDNDKYEGScAEQDGSGWWMNRcHAGHLNGKYYFGGNYRKTD

              .. .......  .... . ....  .  .   . .↓.    . _
Human     TPNGYDNGIIWATWKTRWYSMKKTTMKIIPFNRLTIGEGQQHHLGGAKQAGDV
Lamprey   VEFPYDDGIIWATWHDRWYSLKMTTMKLLPMGRDLSGHGGQQQSKGNSRGDN
                                                  ↑   ‾
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FIGURE 3. Alignment of lamprey and human fibrinogen $\gamma$-chain sequences. There are 205 identities among the 408 aligned residues, which amounts to 50.2% identity (from Strong *et al.*, 1985).

molecule is running smoothly. The data also indicate that the gene duplication which led to $\beta$ and $\gamma$ chains must have occurred about 600 million years ago. The fact that the $\alpha$ chains are more divergent implies that the first duplication, leading to $\alpha$ and non-$\alpha$ chains, may have occurred as much as a billion years ago, although in this case we must be more cautious, since there are portions of fibrinogen $\alpha$ chains that are changing significantly faster than the rest of the molecule, and the elapsed time involved may therefore be significantly less.

In either case, there is a paradox here, for although the sequence data indicate that fibrinogen was evolved somewhere between 600 million and a billion years ago, nobody has yet observed such a molecule among the invertebrates or protochordates, and it is not for a lack of looking. It is true that some arthropods have a protein in their hemolymph that can be gelled under appropriate circumstances, but it is a fundamentally different molecule and does not share ancestry with vertebrate fibrinogen (Fuller and Doolittle, 1971; Doolittle and Fuller, 1972). So, more than 25 years after first asking when and where did the vertebrate fibrinogen molecule evolve, I am still begging the question. The tools for searching are much improved now, however, and we are now using recombinant DNA techniques to probe the genomes of various protochordates and echinoderms. The advantage is that even if the ancestral protein is made in amounts too small to have been detected by ordinary means, or in an intracellular setting where we wouldn't have seen it, we should be able to find its genes with appropriate DNA probes.

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          . ...    .   . ..        .    .   .            . ...     ...
Human     GHRPLDK KREEAPSLRPAPPPISGGGYRARPAKAAATQKKVERKAPDAGGcLHADPDLG
Lamprey   GVRPLPSGTRVRRPPLR  HRRLAPGAVMSRDPPASPRPQEAQKAIRDEGGcMLPESDLG

          ....... . . .. .  . .     .  .                 ..   .
Human     VLcPTGcQLQEALLQQERPIRNSVDELNNNVEAVSQTSSSSFQYMYLLKDLWQKRQKQVK
Lamprey   VLcPTGcELREELLKQRDPVRYKISMLKQNLTYFINSFDRMASDSNTLKQNVQTLRRRLN

                     . .           .     ..   . .. .. . .  .     . . .
Human     DNENVVNEYSSELEKHQLYIDETVNSNIPTNLRVLRSILENLRSKIQKLESDVSAQMEYc
Lamprey   SRSSTHVNAQKEIENRYKEVKIRIESTVAGSLRSMKSVLEHLRAKMQRMEEAIKTQKELc

            .... .  .....  .. . . .. ... . ....    .. . ...    ..... .
Human     RTPcTVScNIPVVSGKEcEEIIRKGGETSEMYLIQPDSSVKPYRVYcDMNTENGGWTVIQ
Lamprey   SAPcTVNcRVPVVSGMHcEDIYRNGGRTSEAYYIQPDLFSEPYKVFcDMESHGGGWTVVQ

          .. ...  . . .  ..  ... .    ..  .  .......     ...       .
Human     NRQDGSVDFGRKWDPYKQGFGNVATNTDGKNYcGLPGEYWLGNDKISQLTRMGPTELLIE
Lamprey   NRVDGSSNFARDWNTYKAEFGNIA FGNGKSIcNIPGEYWLGTKTVHQLTKQHTQQVLFD

          . .. .  . . .  .   ...  .   .  . . .....  .. .... ..........
Human     MEDWKGDKVKAHYGGFTVQNEANKYQISVNKYRGTAGNALMDGASQLMGENRTMTIHNGM
Lamprey   MSDWEGSSVYAQYASFRPENEAQGYRLWVEDYSGNAGNALLEGATQLMGDNRTMTIHNGM

           ... ..... .   .. . .. .. ..................... ..   .  ....
Human     FFSTYDRDNDGWLTSDPRKQcSKEDGGGWWYNRcHAANPNGRYYWGGQYTWDMAKHGTDD
Lamprey   QFSTFDRDNDNWNPGDPTKHcSREDAGGWWYNRcHAANPNGRYYWGGIYTKEQADYGTDD

          ............... . .. ..  .
Human     GVVWMNWKGSWYSMRKMSMKIRPFFPQQ
Lamprey   GVVWMNWKGSWYSMRQMAMKLRPKWP
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FIGURE 4. Alignment of lamprey and human fibrin $\beta$-chain sequences. There are 218 identities among the 443 aligned residues, which amounts to 49.2% identity (from Bohonus *et al.*, 1986).

## ALBUMIN

As I mentioned earlier, not everyone in the comparative plasma protein field is convinced that fish have an albumin in their blood plasma, and it is often viewed as a protein that evolved as a part of the land invasion by vertebrates (again, for a review of opinions, see Doolittle, 1984). Its great abundance was long considered by physiologists as being primarily of osmotic importance ("Starling's Law of Colloid Osmotic Pressure"), even though many investigators recognized its important contributions to the transport of fatty acids and other nonpolar materials.

In the mid-1970's, Jim Brown (1976) reported the amino acid sequence of the bovine albumin molecule, and the data revealed that the protein had experienced a number of internal duplications. In particular, the sequence could be divided into three equivalent regions of about 190 amino acids each. The sequences of two of these were more similar then either was to the third, suggesting that there had been a doubling at one point, from a molecule of about 190 amino acids to one of 380, and then a second, incomplete, duplication that gave rise to the existing 580-residue structure. There was also evidence that the fundamental macrodomain structure (*ca.* 190 residues) had itself evolved as the result of internal duplications from a more primitive sequence of about 77 residues. Comparative studies on the human protein indicated that albumin is changing almost twice as fast as the hemoglobin polypeptides. Brown (1976) interpreted his data as indicating that the tandem duplications

TABLE III

*Comparison of lamprey and mammalian plasma albumins**

| | Mammalian | Lamprey |
|---|---|---|
| Molecular weight | 69,000 | 175,000 |
| Water-soluble | Yes | Yes |
| Bind bromphenyl blue | Yes | Yes |
| Free-SH | 1 | 2 |
| Tryptophans | 2–3 | 3–4 |
| Disulfides | Rich | Rich |

* From Kuyas *et al.* (1983).

leading to the three macrodomains occurred about 700 million years ago, a number that may be a little high, but one that obviously suggests that invertebrates ought to have albumin. Again, no one has ever observed a protein among the invertebrates that bears any resemblance to a vertebrate albumin.

My own calculation of when the albumin internal duplications ought to have occurred resulted in a somewhat more recent time than Brown had reckoned, and, accordingly, I considered the possibility that fish, and the lamprey in particular, might have a smaller, more ancient, molecule, corresponding to the one- or two-macrodomain stage. Imagine our surprise, then, when we found that one of the most abundant proteins in lamprey plasma has all the properties of a mammalian albumin (Table III) except that its molecular weight is 175,000, fully two and-a-half times *larger* than the protein found in terrestrial vertebrates! There are two lessons here, the more important of which is that we must always remember that in any divergence there are opportunities for change along both lines of descent. Clearly the lamprey albumin has experienced a number of further internal duplications since cyclostomes and other vertebrates diverged. The second lesson is a reminder of another principle in protein evolution: Duplication begets more duplication. This has to do with the increased opportunities for DNA mis-matching between similar sequences. These in turn can lead to more unequal crossing over, for example (for a fuller discussion, see Doolittle, 1979). We are currently trying to clone the lamprey albumin in order to obtain its complete sequence. With that we should be able to pinpoint precisely when the first duplications took place, events that must pre-date the divergence of cyclostomes and other vertebrates. Like fibrinogen, albumin molecules ought to exist among the protochordates and some invertebrates.

## APOLIPOPROTEINS

During our studies on lamprey albumin, we uncovered two small polypeptide contaminants that turned out to be high density apolipoproteins. In fact, these two proteins are among the most abundant proteins in lamprey plasma (Fig. 5). We were able to clone both of them from a lamprey liver cDNA library. Their complete sequences were determined, the larger, composed of 168 amino acids, by Manuel Pontes, a graduate student in our laboratory, and the smaller one, amounting to 76 residues, by Dr. Xun Xu, a visitor from China (Pontes *et al.,* 1987). These two proteins have many of the attributes of the high density apolipoproteins found in mammalian plasma, including structures that are highly helix-permissive and that lack cysteine. Although we were able to align the sequences with some mammalian apolipoprot-

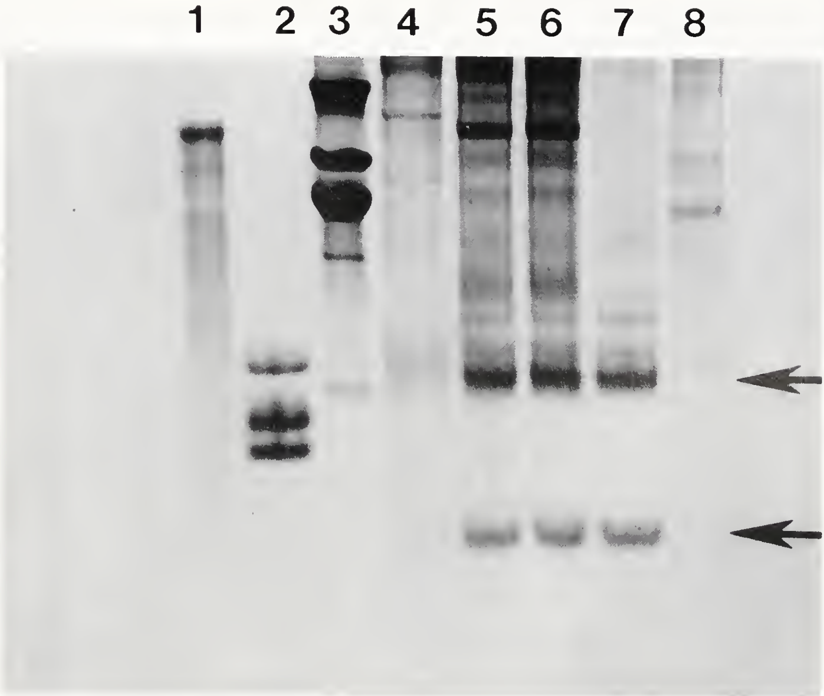

FIGURE 5. SDS-polyacrylamide (7.5%) gel electrophoresis (reducing conditions) of lamprey plasma and plasma materials. 1, purified lamprey transferrin; 2, reference substances (chymotrypsinogen, myoglobin and lysozyme); 3, lamprey fibrinogen; 4, lamprey albumin; 5 and 6, lamprey plasma; 7, high density ultracentrifugate (HDL layer); 8, lipoprotein "cake" from ultracentrifuged lamprey plasma (from Pontes *et al.*, 1987).

eins, the high rate of sequence change in these materials makes it difficult to prove a case for common ancestry.

## TRANSFERRIN

Transferrin, the iron transport protein, is well known to be a major component in the blood plasmas of all vertebrates. It is also common knowledge that among all classes of vertebrates, including cyclostomes, the molecule is an internal dimer with two iron-binding sites. One of our graduate students, Barbara Evans, purified lamprey transferrin, and, together with Dr. Kenneth Watt, determined the amino-terminal 47 amino acids (Evans *et al.*, 1984). Attempts to clone the message were unsuccessful, however. We had hoped to shed more light on when the tandem duplication occurred that led to the double-sized molecule. In the meantime, an iron-binding protein has been found in the ascidian *Pyura stolonifera* that is half the size of vertebrate transferrin (Huebers *et al.*, 1984). It will be of great interest to see if the sequence of the ascidian protein is recognizably homologous with the vertebrate molecule.

## COMPUTER SEARCHING

Up to this point, I have been discussing the evolution of vertebrate plasma proteins on the basis of comparisons of proteins as they exist in contemporary mammals on the one hand, and in lampreys, the most primitive of the vertebrates, on the other. I'd like now to turn to another approach, one in which the principal tool is the computer. The basic idea is to look for relationships between and among proteins simply on the basis of their amino acid sequences. Let me start with one of my favorite examples. A few years ago, the amino acid sequence of rat angiotensinogen was published. Angiotensinogen is the precursor of the hormone angiotensin, a 10-residue peptide that is critical for the maintenance of water balance. Interestingly, the precursor of this short peptide is enormous, amounting to no less than 453 amino acids in length. Nevertheless, I typed the entire sequence into my computer and searched it against a data base I was maintaining. Unexpectedly, the computer reported that angiotensinogen is related to $\alpha$-1-antitrypsin (Doolittle, 1983). Overall, the two proteins were only a little more than 20% identical, but the fact that the resemblance extended over the course of almost 400 residues made the match-up quite significant. Now, $\alpha$-1-antitrypsin is a protease inhibitor that is one of the abundant proteins in blood plasma (Table I), and the fact that it could share common ancestry with a polypeptide hormone precursor was astonishing to me. It was already known that $\alpha$-1-antitrypsin is related to several other protease inhibitors, and since that time more inhibitor members of the family, which are now referred to as "serpins," have been identified. But the utilization of a large, apparently derelict, protease-inhibitor as the precursor of a tiny polypeptide hormone remains a unique example of the re-utilization of good stable proteins in new settings with quite different functional demands.

Since that time many other unexpected match-ups have resulted from the computer-searching of new sequences through data banks, and many of these have involved the vertebrate plasma proteins. The blood coagulation Factors VIII and V, for example, which are themselves homologous, were found to share common ancestry with the copper-binding protein ceruloplasmin (Vehar *et al.,* 1984). Less unexpectedly, for those following the structure and functional relationships of the plasma proteins, was the report that $\alpha$-2-macroglobulin is homologous with the complement components C3 and C4 (Sottrup-Jensen *et al.,* 1985). Indeed, I myself had previously predicted this relationship on the basis of partial sequences and other features these molecules have in common (Fig. 6).

The sequence of $\alpha$-2-macroglobulin, which appears to be the root-ancestor of the group, is interesting on other grounds. It is a very long sequence, consisting of over 1450 amino acid residues, and yet there are no residual signs of past internal duplications. The ordinary way for proteins to become elongated, as we have noted previously for albumin and transferrin, is by internal duplication. The absence of any vestige of internal duplication in $\alpha$-2-macroglobulin implies that it is either very old or is changing very fast, or both. As it happens, yesterday afternoon I met Jim Quigley out in front of the MBL, and he told me that he and Peter Armstrong have purified a homologue of $\alpha$-2-macroglobulin from the hemolymph of the horseshoe crab (Quigley and Armstrong, 1985). It will be very interesting to see what the sequence of the *Limulus* protein is like compared with the mammalian counterparts. At the very least the matter of its rate of change should be settled.

Unanticipated sequence resemblances among the plasma proteins continue to mount. The vitamin D-binding protein known as the "group-specific component" has recently been found to be related to albumin, the sequences being 24% identical (Yang *et al.,* 1985). Further, some plasma proteins whose functions had not previously been known, are now being classified on the basis of their relationship to other proteins. A minor component known as the "gamma-trace protein" is clearly related to the kininogen family, and kininogens in turn are now known to be related to thiol protease-inhibitors (Doolittle, 1985b; Muller-Esterl *et al.,* 1985).

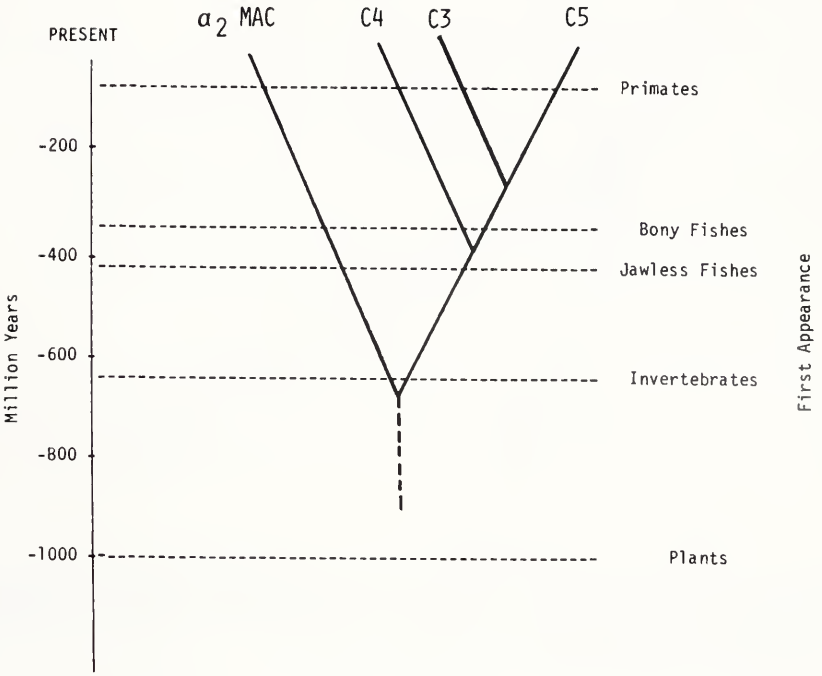

FIGURE 6. Possible scheme of gene duplications leading to present-day α-2-macroglobulin and three complement proteins. There are fewer species comparison data available for this tree than there are for the hemoglobin and fibrinogen trees in Figures 1 and 2, and, as a result, the divergence times are not nearly as accurate (from Doolittle, 1984)

## EXON SHUFFLING

The resemblances that are being uncovered do not always extend over the full lengths of two similarly sized proteins. Sometimes, in fact, two proteins will have obviously similar segments over a portion of their lengths, and then, abruptly, the similarity is lost. In some cases one of the sequences may even switch to looking like a part of some third protein. One such case is observed in the protein known as tissue plasminogen activator (TPA). This protease precursor has short segments near its amino terminus that resemble, successively, fibronectin, epidermal growth factor, and prothrombin (Banyai *et al.*, 1983).

At about the same time the curious mosaic form of TPA was noticed, the full sequence of the epidermal growth factor precursor was reported. When we searched this very long sequence (more than 1200 amino acid residues) against our sequence collection, we were surprised to find that the candidate sequences retrieved were all blood clotting factors: Factor IX, Factor X, and Protein C (Doolittle *et al.*, 1984). The resemblances were limited to two 40–45 residue segments in the clotting proteins, but 10 similar segments appeared in the EGF-precursor (Fig. 7). Curiously, four of the EGF-precursor segments were more closely related to one of the segments in the clotting factors, while the six others, including EGF itself, were more closely related to the second. This isn't what one expects from a simple homologous crossing over; rather, it suggests repeated exchanges (Doolittle *et al.*, 1984).

Shortly thereafter, the laboratory of Brown and Goldstein reported the sequence

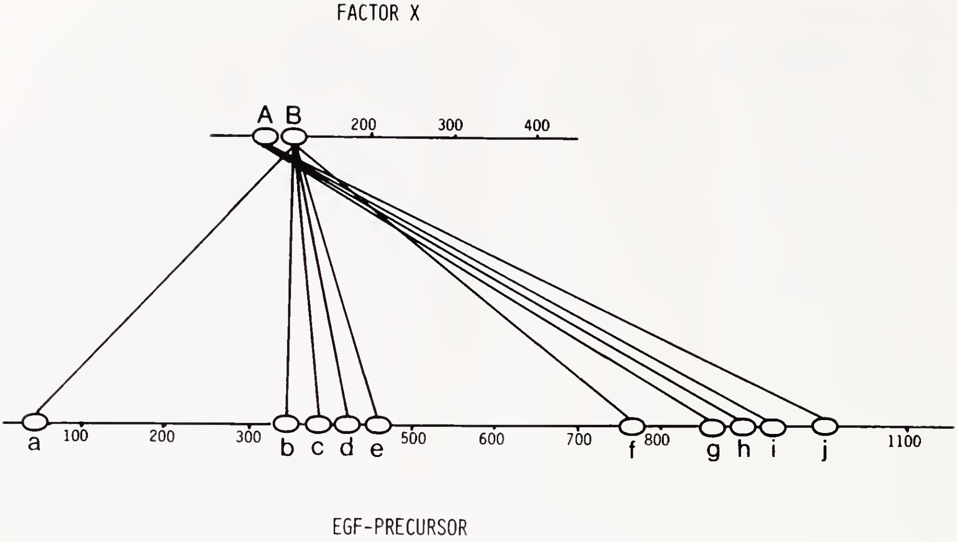

FIGURE 7. Schematic depiction of resemblances between segments found in the blood clotting protein Factor X and the membrane-associated protein which is the epidermal growth factor (EGF) precursor. Each of the oval segments represents a polypeptide segment of 40–45 residues, most of which contain six cysteines. EGF itself is represented by the right-most oval in EGFP.

of the LDL-receptor (Sudhoff *et al.*, 1985). Astonishingly, this receptor protein had a portion of about 200 amino acids that were strikingly similar to a segment of the EGF-precursor (Fig. 8). Moreover, another long region had seven repeated segments, the sequences of which were all similar to two segments found in the complement protein C9 (Fig. 8). Obviously DNA was being swapped around in a way that was putting similar peptide segments in various proteins (Table V).

The major clue for explaining how these exchanges must be occurring originally came from Ny *et al.* (1984) who determined the genomic DNA sequence corresponding to TPA. What they found was that the segments corresponding to other protein types, as identified by Banyai *et al.* (1983)—fibronectin, EGF, and prothrombin—were separated in each case by introns. Subsequently other workers found virtually the same pattern in the genes of the EGF-precursor and the LDL-receptor. In other words, each of the symbols corresponding to one of the prototype segments in Figure 8 actually corresponds to an exon. In these cases, exons must actually correspond to independently folding domains that can be shuffled about from protein to protein without disrupting the rest of the protein structure.

So far, about a half dozen such exchangable modules have been identified. They range in size from 40–80 amino acid residues. The most recent one to be characterized has already been found in several plasma proteins, including $\beta$-2-glycoprotein, complement factor B and complement factor H (Ripoche *et al.*, 1986). Moreover, at a recent Cold Spring Harbor meeting I learned from Earl Davie that the Factor XIII b-chain also contains a long series of segments of this type (E. Davie, pers. comm.).

## OVERVIEW

It appears that the proliferation of vertebrate plasma proteins has been due to two related but distinguishably different phenomena. The first is orthodox gene duplica-

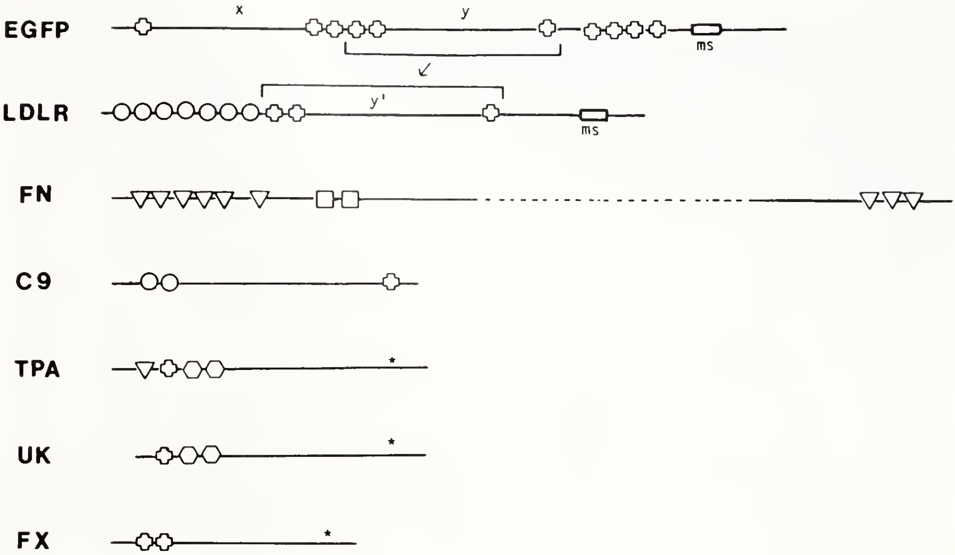

FIGURE 8. Schematic comparison of several vertebrate proteins that contain homologous "modules" resulting from exon shuffling. EGFP, epidermal growth factor precursor; LDLR, low-density lipoprotein receptor; FN, fibronectin; C9, complement component 9; TPA, tissue plasminogen activator; UK, urokinase; FX, blood clotting Factor X. The different types of peptide segments are denoted by the different symbols, asterisks (*) are the active sites of serine proteases, and "ms" marks membrane-spanning segments (from Doolittle, 1985a).

tion, complete versions of which can give rise to new, modified gene products, while partial or incomplete versions give rise to elongated, internally duplicated sequences. The second proliferative force is exon shuffling, an operation that has led to a relatively small set of polypeptide segments being present in many different proteins (Table IV). Most of these segments likely act as recognition units for binding the proteins

TABLE IV

*Some animal proteins that have sequence segments in common*

*A. EGF-type:*
Epidermal growth factor precursor
Tumor growth factors
Low density lipoprotein receptor
Factor IX
Factor X
Protein C
Tissue plasminogen activator
Urokinase
Complement C9
Notch protein (Drosophila)
Lin-12 (Nematode)
Thrombospondin

*B. Fibronectin "finger":*
Fibronectin
Tissue plasminogen activator

*C. C9-type:*
Complement C9
Low density lipoprotein receptor
(Fibronectin?)
Notch (Drosophila)
Lin-12 (Nematode)

*D. Proprotease "kringle":*
Plasminogen
Tissue plasminogen activator
Urokinase
Prothrombin

*E. β-2 type:*
$\beta_2$-glycoprotein
Complement Factor B
Complement Factor H
Factor XIII b-chain

TABLE V

*Some known protein families found in vertebrate blood plasma*

- A. Albumin
  - $\alpha$-fetoprotein (fetal albumin)
  - Vitamin-D-binding protein (group-specific component)
- B. Immunoglobulins (assorted)
  - $\beta_2$-microglobulin
- C. Fibrinogen $\alpha$ chain
  - Fibrinogen $\beta$ chain
  - Fibrinogen $\gamma$ chain
- D. Coagulation serine proteases
  - Fibrinolysis serine proteases
  - Complement serine proteases
  - Miscellaneous serine proteases
  - Haptoglobin
- E. $\alpha_2$-macroglobulin
  - Pregnancy zone protein
  - Complement C3
  - Complement C4
  - Complement C5
- F. $\beta_2$-Glycoprotein
  - Complement Factor B (nonenzyme portion)
  - Complement Factor H
  - Factor XIII b-chain
- G. Retinol-binding protein
  - $\alpha_1$-microglobulin
- H. Antithrombin III
  - $\alpha_1$-antitrypsin
  - $\alpha_1$-antichymotrypsin
  - Angiotensinogen
- I. Ceruloplasmin
  - Factor V
  - Factor VIII
- J. Lipid-binding proteins
  - (A, B, C, etc.)
- K. Kininogens
  - $\beta_1$-Microglobulin
  - Acute phase proteins
  - Thiol protease inhibitors
- L. Transthyretin (prealbumin)
  - Glucagon
  - Glycentin
- M. $\beta$-Thromboglobulin
  - Platelet factor 4
- N. Serum amyloid P-component
  - C-reactive component

differentially to various cells. In any case, the net result is that the six hundred electrophoresis components observed in mammalian plasma will ultimately be grouped into a relatively small number of families (Table V).

When did all these events transpire? Doubtless they occurred throughout the history of the vertebrates, but the fact is that much of the inventive action must have been a necessary precondition to the evolution of vertebrates themselves. Many of the sequence comparisons we have discussed this evening indicate that the original gene duplication leading to some of the principal plasma proteins must have occurred among invertebrate ancestors. The point is made further by the fact that the lamprey, one of our most distant vertebrate relatives, has so many plasma proteins in common with mammals. The fact that many of these proteins have not yet been identified among invertebrates or protochordates remains a mystery, but it also provides an opportunity for much more exploration. I have no doubt that that matter will resolve itself as more sequence data are collected, particularly among the protochordates and echinoderms.

## ACKNOWLEDGMENTS

I am grateful to all the many students and associates who have worked with me on these problems over the past 25 years. I thank Karen Anderson for assistance in preparing this manuscript.

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# THE LARVAL STAGES OF *LITHOTRYA DORSALIS* (ELLIS & SOLANDER, 1786): A BURROWING THORACICAN BARNACLE

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## ABSTRACT

*Lithotrya dorsalis* is a member of the only genus of thoracican barnacles known to burrow and is widely distributed throughout the tropical western Atlantic. It occurs primarily in high energy intertidal environments. *L. dorsalis* undergoes the typical thoracican larval sequence. Six naupliar stages are followed by the cyprid stage. These larvae were reared in the laboratory and their stages are described for the first time. Newly hatched stage I nauplii are typically 360 μm in total length; larval size increases to 1100 μm by the 6th instar. The most distinguishing characteristic of *Lihtotrya dorsalis* nauplii is the presence of unusually long, spinulated posterior shield spines in stages IV through VI. Complete larval development (stage I nauplius to cyprid) averaged 18 days and ranged from 12 to 23 days. Scanning electron micrograph descriptions of the cyprid cuticle of this animal, showing a unique striated appearance, are also included.

## INTRODUCTION

First described by Sowerby (1822), the genus *Lithotrya* (family Scalpellidae, subfamily Lithotryinae) is further treated by Darwin (1851), Sewell (1926), Otter (1929), and Cannon (1947). *L. dorsalis* is considered to be the only species occurring in the western Atlantic (Zevina, 1981). Found exclusively in carbonate substrata, this species is an abundant and ubiquitous constituent of exposed tropical coastlines (Ginsburg, 1953; Newell *et al.*, 1959; Ahr and Stanton, 1973; Southward, 1975; Focke, 1977; Spivey, 1981). Settling gregariously, *L. dorsalis* is capable of substantial bioerosion. Bore holes up to 7 cm in length, with a typical oval aperture as large as 5 × 8 mm have been reported (Ahr and Stanton, 1973). These authors described average boring densities in limestone beach rock off Puerto Rico to be one tube per cm$^2$ of rock with up to 30% of rock excavated in some samples. It has been found subtidally (75–80 ft) (Pequegnat and Ray, 1974) and in fringing reef environments (MacGeachy and Stearn, 1976). However, its larval history has remained virtually unknown.

Complete larval descriptions of pedunculate barnacles are listed by Lang (1976). Dalley (1984) has since successfully reared the naupliar stages of *Conchoderma auritum* (L.). Of these pedunculate barnacles, only four are scalpellids (Thoracica: Scalpellidae): *Capitullum mitella* L. (= *Mitella mitella* = *Pollicipes mitella*) (Yasugi, 1937); *Calantica spinosa* Quoy and Gaimard (= *Pollicipes spinosus*) (Batham, 1946); *Scalpellum scalpellum* L. (Kaufmann, 1965); and *Pollicipes polymerus* Sowerby (Lewis, 1975). The burrowing habit of *Lithotrya* makes it unique not only among the scalpellids but among the thoracican cirripeds in general.

Description of the larval stages of *L. dorsalis* make it possible to identify and stage specimens from tropical plankton tows, thereby enhancing future ecological studies.

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Furthermore, documentation of the plate ontogeny and maturation of the post-cyprid, a direct consequence of this study, may have implications for the geological age of the Lithotryinae and the burrowing rate of newly settled cyprids, respectively.

Cyprid appearance is often cited in the literature as being unhelpful in differentiating species because of its interspecific similarity and intraspecific variability in size. Standing (1980) compared seven species of barnacle cyprids at the light microscopy level and concluded that shape (side view) is a more reliable indicator of species than size and further concluded that carapace sculpturing may prove to be a useful tool for differentiating species at the cyprid level. Carapace sculpturing, along with size, was previously used to differentiate cyprids of *Balanus glandula* from *Balanus cariosus*, respectively (Strathman and Branscomb, 1979). Both Lang (1979) and Standing (1980) alluded to the fact that the use of the scanning electron microscope (SEM) may be a valuable tool in extending our knowledge of the external morphological features of the cyprid larva and thereby rendering these features of some diagnostic value at the interspecific level.

SEM descriptions of the external cuticle of the cyprid larva of *L. dorsalis* are included here in an attempt to encourage similar descriptions of this significant stage in future larval studies.

Complete sets of larvae are deposited with the American Museum of Natural History, New York, New York; and the National Museum of Natural History, Washington, DC.

## MATERIALS AND METHODS

Sampling took place in September and October, 1983, and again, at the same time of year, in 1984. Using a hammer and chisel, pieces of limestone reef rock containing adult barnacles were removed from the intertidal zone of Indian Key, Florida, and placed in buckets containing ambient seawater. This rock was returned to the laboratory the same day and vigorously aerated. Over the next several days, the rock was further chiseled into smaller pieces. Gravid individuals, easily recognized by the bright orange pigmentation of the ovigerous lamellae (paired egg bearing structures) seen through the peduncular cuticle, were removed intact and placed individually into small culture bowls. The capitulum was subsequently disjoined and the lamellae were coaxed from the peduncle by applying gentle pressure.

### *Culturing techniques*

The basic rearing techniques of Freiberger and Cologer (1966) were followed. Depending on the degree of maturation, stage I nauplii would either hatch immediately or after incubation of the lamellae for up to 10 days. Maturation in incubated cultures was gauged by the increasing darkness of the naupliar eyespot. Incubating lamellae were placed in 0.45 $\mu$m millipore filtered seawater (MFSW), 30 ppt salinity at 30°C, in the dark. Cultures were observed and seawater was changed daily. Stage I nauplii were transferred at varying densities (2–8 nauplii per ml medium) to stendor dishes containing MFSW reduced to 28 ppt salinity. Cultures were placed in a temperature—controlled chamber at 30°C on a shake table at gentle speed. Either a 12:12 or 18:6 light:dark schedule was used. Various algal diets were tried but the complete larval sequence was only obtained using the mixed algal diets of *Thalassiosira fluviatilus/Tetraselmis suecica* or *Chaetoceros gracilis/Isochrysis* sp. Algae used as food were grown in f/2 medium in batch culture, centrifuged, resuspended in MFSW, and dispensed at initial densities of $2 \times 10^5$ cells/ml. The antibiotics Streptomycin sulfate, Polymixin B sulfate (both at 12 $\mu$g/ml) and Penicillin G (25 $\mu$g/ml) (Sigma) were

TABLE I

Lithotrya dorsalis: *Measurements of larvae**

| | Total length (μm) | | | Shield length (μm) | | | Width (μm) | | |
|---|---|---|---|---|---|---|---|---|---|
| Stage | x̄ | SD | Range | x̄ | SD | Range | x̄ | SD | Range |
| I | 362 | 25 | 333–421 | | | | 186 | 15 | 170–219 |
| II | 546 | 10 | 540–564 | 202 | 9 | 190–220 | 238 | 14 | 220–263 |
| III | 749 | 35 | 681–795 | 243 | 13 | 220–257 | 258 | 10 | 237–267 |
| IV | 899 | 44 | 799–952 | 345 | 17 | 303–360 | 323 | 17 | 284–335 |
| V | 1035 | 40 | 936–1070 | 446 | 13 | 435–472 | 409 | 19 | 366–438 |
| VI | 1141 | 37 | 1070–1171 | 510 | 15 | 484–536 | 464 | 22 | 424–493 |
| cyprid | 518 | 15 | 496–542 | | | | 240 | 8 | 233–252 |

* n = 10 in stages I–V and cyprid, n = 8 in stage VI.
x̄ = mean; SD = standard deviation.

used (Landau and D'Agostano, 1977). Glassware was autoclaved daily. Cultures were transferred by pipet every 24 h. Several individuals from each culture were removed daily and preserved in either 70% ethanol or 5% buffered formalin in seawater. An ocular micrometer was used to approximate the stage of these individuals. Representatives from each stage were later stained with chlorazol black in a 10% solution of glycerin in 70% ethanol. The alcohol was allowed to slowly evaporate. Larvae, now more manipulable, were then either mounted whole or dissected with fine tungsten needles. Stages were verified and drawings were made using camera lucida on a Wild M20 compound microscope.

### *Specimen preparation—SEM*

Cyprids prepared for scanning electron microscopy were initially fixed in buffered glutaraldehyde and dehydrated through a graded series of ethanols. Then the cyprids were either placed in trichlorotriflouroethane (Nott, 1969) which was allowed to slowly evaporate under a bell jar through which nitrogen gas was gently bubbled, or were transferred to acetone and then critical point dried (Samdri pvt.3, Tousimis). Using a method similar to Waller (1981), these larvae were individually glued with polyvinyl acetate to one end of a small platinum wire. The other end of this wire was twisted into a loop which was then attached to a glass cover slip using silver paint. The cover slip was then affixed to a mounting stub using double sided tape. The animals were then sputter coated with gold/palladium under vacuum (Hummer 5, Technics). This method allows manipulation of the specimen by bending the wire with fine forceps. Larvae treated this way were examined with either a JSM-U3, JEOL or a 1000A, AMRay scanning electron microscope at a gun potential of 15 to 20 kV.

## RESULTS

### *Larval cultures*

*Lithotrya dorsalis* exhibited the typical thoracican larval sequence. Six naupliar stages were followed by the cyprid stage. Average survival from naupliar stage I to cyprid in 29 larval cultures was 12.5% (range = .54–46.1%). Cultures with initially lower densities (1–2 nauplii/ml medium) yielded higher percent survival. Complete larval development (stage N I to cyprid) averaged 18 days and ranged from 12 to 23 days. Several cultures molted in synchrony, the majority did not. Sizes of larval stages are given in Table I. Shield lengths were approximated for naupliar stages II and III because of the lack of a distinct posterior margin in these stages.

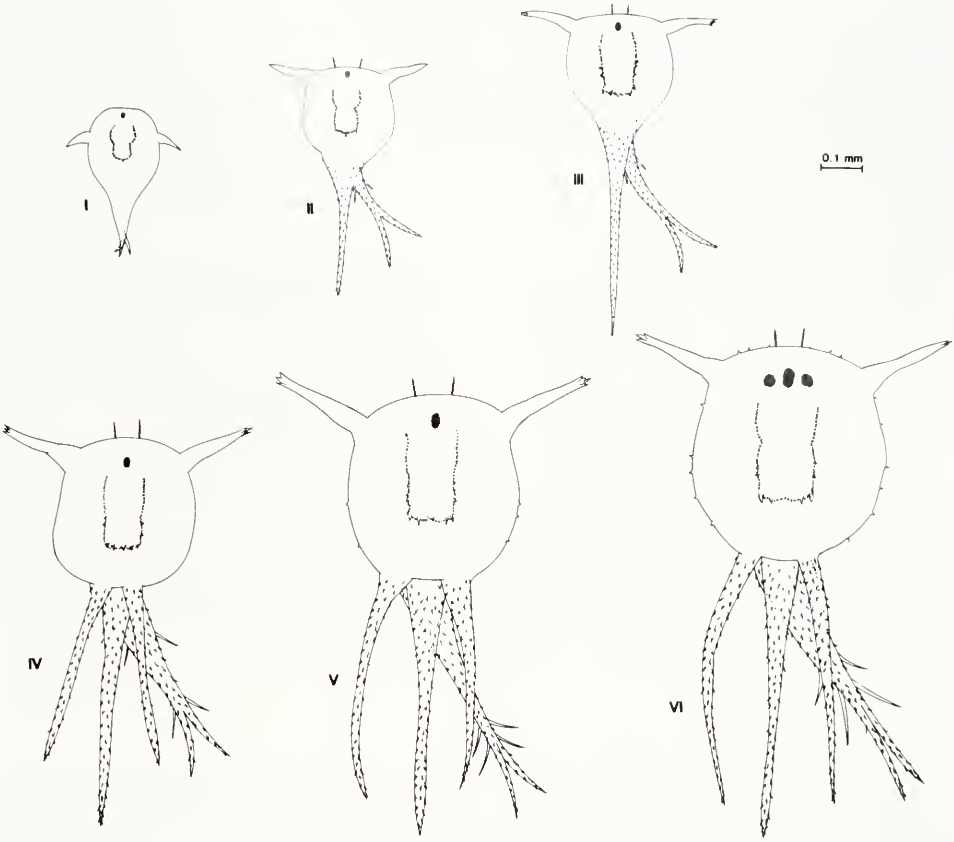

FIGURE 1. *Lithotrya dorsalis.* Dorsal view of shield outlines of naupliar stages I–VI.

### *The nauplii*[1]

The naupliar stages as seen from above are illustrated in Figure 1. First stage nauplii (Fig. 1, I) are characterized by frontolateral horns directed posteriorly with the posterior region of the cephalic shield tapering distinctly into a rudimentary thoracic spine dorsal to a small, forked thoraco-abdominal process. A median naupliar eye is evident and remains throughout naupliar development. A unilobed labrum with one median tooth is present. Stage I nauplii have the full complement of naupliar appendages, the uniramous antennules, biramous antennae, and mandibles, provided with simple setae. It is a nonfeeding stage and molts to stage II within one or two hours post hatching.

The thoraco-abdominal process and dorsal thoracic spine become elongated and spinulated at stage II (Fig. 1, II) and remain this way throughout naupliar development. The thoraco-abdominal process is distinctly and deeply forked and contains one pair of series 1 spines (Fig, 2, II). A row of fine, slightly arched hairs appear on each side of the proximal portion of the labrum. An additional tooth appears at either end of the posteriolateral margin (Fig. 3, II). Frontal filaments are apparent at stages II–VI.

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[1] Descriptive terminology after Lang (1979) with the exception of the term "thoraco-abdominal" process suggested by W. A. Newman.

Stage III nauplii (Fig. 1, III) are somewhat similar to stage II nauplii but the thoraco-abdominal process and dorsal thoracic spine have become proportionately longer relative to the cephalic shield. The posterior margin of the labrum now bears five teeth and two to three spines appear laterally (Fig. 3, III). As is typical of cirriped nauplii in general, the frontolateral horns at stages III–VI appear split at the tip. Stage III nauplii appeared in culture as early as day 3.

Stage IV nauplii (Fig. 1, IV) are easily distinguished from previous stages by the presence of unusually long, barbed posterior shield spines and a distinct posterior shield margin. In active, swimming larvae, the posterior shield spines, present throughout stages IV–VI, curve prominently away from the main body axis. One pair of series 1 spines as well as 1 median and 1 pair of series 2 spines (Fig. 2, IV) are evident on the thoracic portion of the thoraco-abdominal process of stage IV nauplii [Brown and Roughgarden (1985) refer to series 2 spines as thoracic spines.]

Increasing proportionately in size, stage V nauplii (Fig. 1, V) possess several spines on the lateral posterior margin of the cephalic shield. One pair each of series 1 and 3 spines as well as one median and two pairs of series 2 spines are present on the abdominal process (Fig. 2 V). The earliest day of appearance for stage V nauplii was day 7.

Approximately 24 hours post-molt, a pair of compound eyes become visible in stage VI nauplii (Fig. 1, VI). A variable number of spines are present on the anterior as well as lateral margins of the cephalic shield. Six pairs of series 2 spines and one pair each of series 1 and 3 spines are present on the thoraco-abdominal process (Fig. 2, VI).

### *Thoraco-abdominal process and caudal spines*

The thoraco-abdominal process and corresponding caudal spines for naupliar stages I–VI are shown in relation to the dorsal thoracic spine and posterior shield spines in Figure 2. The thoraco-abdominal process in all stages is shorter than the dorsal thoracic spine. The distal end of the thoraco-abdominal process is deeply forked in naupliar stages II–VI. The appearance of caudal spine series 1, 2, and 3 on this process closely follows the sequence established for other cirripeds (Moyse, 1961; Lang, 1979). However, examination of several stage V nauplii revealed 3 pairs of series 2 spines instead of the usual 2 as drawn. The thoraco-abdominal process along with the dorsal thoracic spine (stages II–VI) and the posterior shield spines (stage IV–VI) are heavily spinulated. There appears to be no pattern to this secondary ornamentation.

### *Labrum*

The unilobed labrum (Fig. 3), characteristic of all lepadomorph nauplii, is distinctly indented at approximately midlength in naupliar stages I and II of *Lithotrya dorsalis* (Fig. 3, I & II). The proximal half of the stage I labrum is noticeably wider than the distal portion. By naupliar stage III, the median tooth now appears clearly associated with the labral gland (Fig. 3, III). Two to three spines are now present on the lateral edges. On the dorsal (inside) surface a row of very fine hair-like processes appear along either side of the labral gland duct. At stages IV–VI (Fig. 3, IV, V, & VI), very thick, hair-like projections appear to surround all but the median tooth on the posterior margin. By stage V, the median tooth becomes less prominent while the remaining teeth become more pronounced. By stage VI, the median tooth is reduced to an inconspicuous knob and appears disassociated from the now indistinct labral gland.

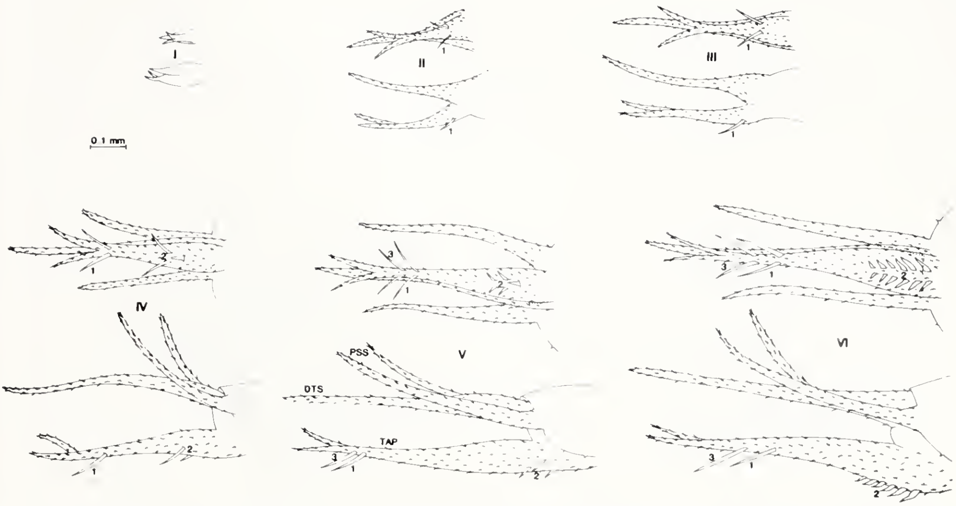

FIGURE 2. *Lithotrya dorsalis.* Ventral and lateral views of the dorsal thoracic spine (DTS) and thoraco-abdominal process (TAP) relative to the posterior shield spines (PSS) in naupliar stages I–VI. Caudal spine series are designated by Arabic Numbers (1, 2, and 3).

## *The appendages*

The uniramous antennules (Fig. 4), biramous antennae (Fig. 5), and mandibles (Fig. 6) are shown for all 6 naupliar stages. The entire setae are not drawn, in most cases, due to their extremely long and delicate nature. Using the basic setation formulae of Newman (1965) as modified by Lang (1979), these appendages are further described in Table II.

The number of setae appearing within a particular stage was relatively constant, reinforcing the idea that setation of an appendage (barring the consequences of dissection) is an accurate indication of naupliar stage (Lang, 1979; Dalley, 1984). The transition from simple to plumose setae appeared to be somewhat variable, particularly on the antennal and mandibular endopodites of stages IV–VI.

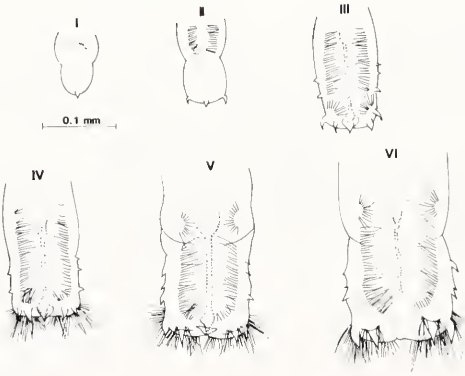

FIGURE 3. *Lithotrya dorsalis.* Labrum of naupliar stages I–VI.

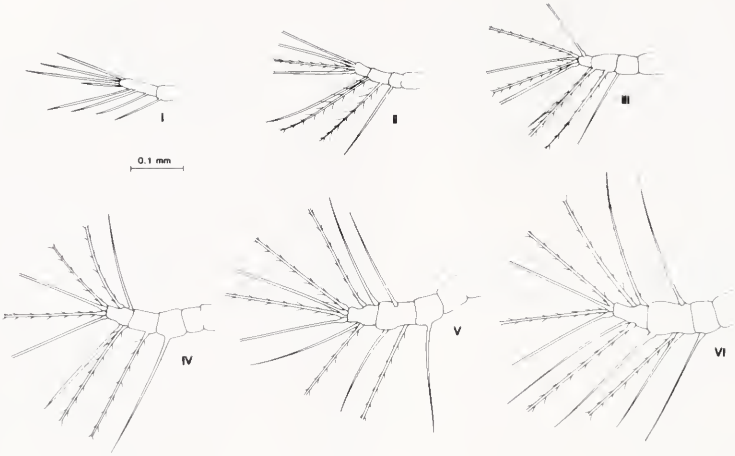

FIGURE 4. *Lithotrya dorsalis*. Antennule of naupliar stages I–VI.

### *The cyprid*

The cyprid (Fig. 7A) is relatively small (averaging 518 μm total length) and similar in overall appearance to other previously described cyprid larvae at the level of light microscopy. A bivalved carapace, compound and median eyes, six pairs of biramous natatory appendages (Fig. 7B), caudal furca (Fig. 7C), and a pair of antennules (Fig. 7D) modified to act as exploratory and attachment organs are present. The anterior margin of the cyprid is evenly rounded. The posterior ventral margin tapers more sharply than the dorsal margin. As a consequence, the posterior end appears more ovoid (Fig. 7A). The living cyprid is somewhat translucent in both the anterior and posterior extremities. The bulk of the body is filled with golden-colored oil cells. There is an irregularly shaped, orange pigment area in the mid to posterior region. The compound eyes have a distinct dark red coloration. The setation of the cyprid appendages (Fig. 7B, C, D) is remarkably consistent with that described for other species (Batham, 1946; Walker and Lee, 1976).

The use of the scanning electron microscope in describing barnacle cyprids has been limited. The cyprid antennule of *Semibalanus balanoides* L. was described by Nott (1969). Walker and Lee (1976) studied the surface structure of the *S. balanoides* cyprid using SEM. Svane (1986) showed a settling *Scalpellum scalpellum* cyprid (low magnification) using SEM. Walker (1985) described the external features of the cyprid of the rhizocephalan, *Sacculina carcini* Thompson. Other rhizocephalan cyprids described with the SEM are *Lernaeodiscus porcellanae* (Ritchie and Høeg, 1981; Høeg, 1985a) and *Clistosaccus paguri* (Høeg, 1985b).

It is perhaps premature to speculate about the reliability of cyprid carapace sculpturing as revealed by the SEM in differentiating species until more such descriptions are available for meaningful comparisons. But at the very least, such SEM descriptions should supplement other characteristics (size, shape, color) currently being used with varying amounts of success.

SEM investigation of the cyprid larva of *Lithotrya dorsalis* reveals distinct sculpturing over the entire carapace surface (Fig. 8A, C; 9A). Sculpturing consists of carinae arranged in a somewhat parallel configuration and interspersed with numerous cuticular setae (Fig. 9B), pores (Fig. 9C) and small papillae (Fig. 9D). The parallel

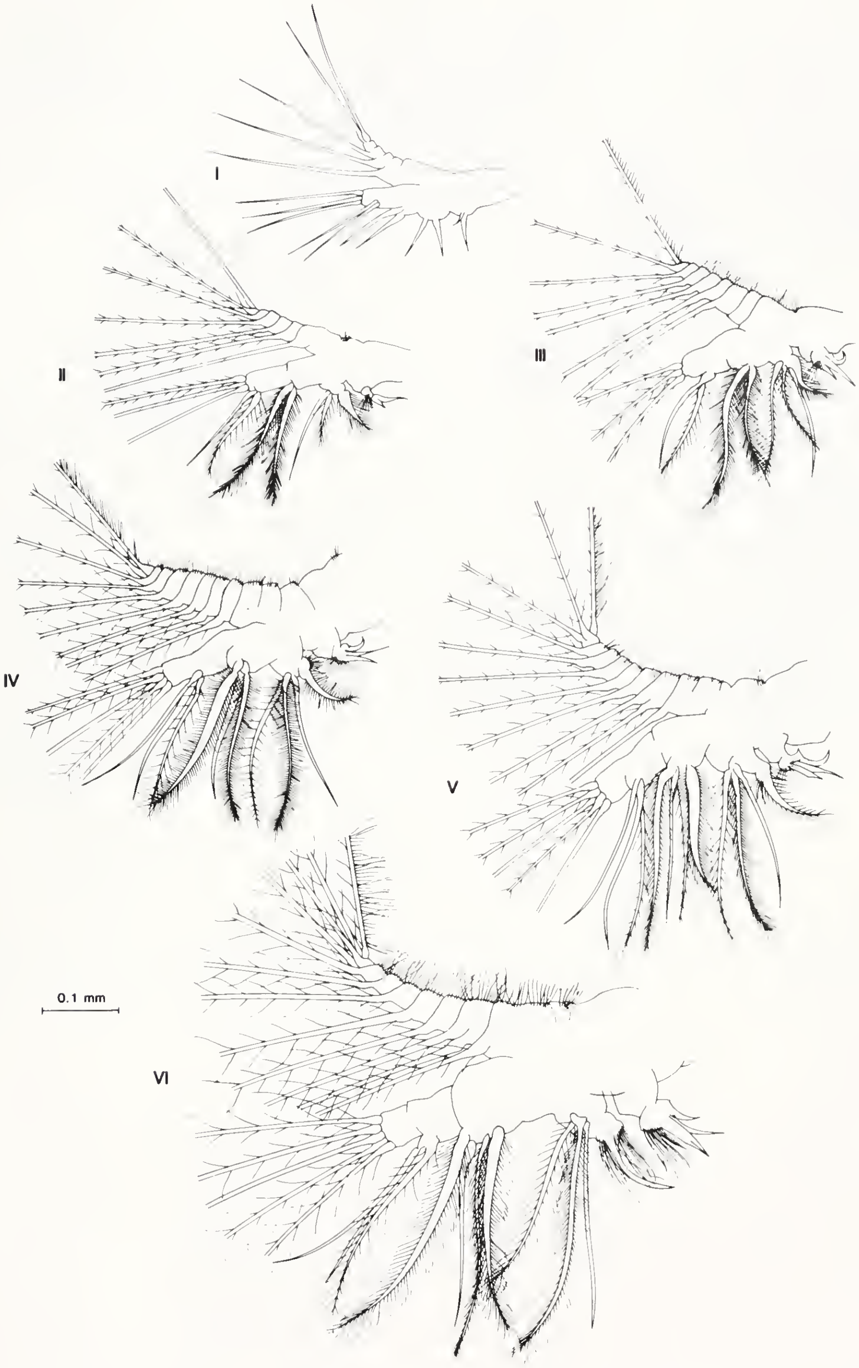
I
II
III
IV
V
VI
0.1 mm

FIGURE 5. *Lithotrya dorsalis.* Antenna of naupliar stages I–VI.

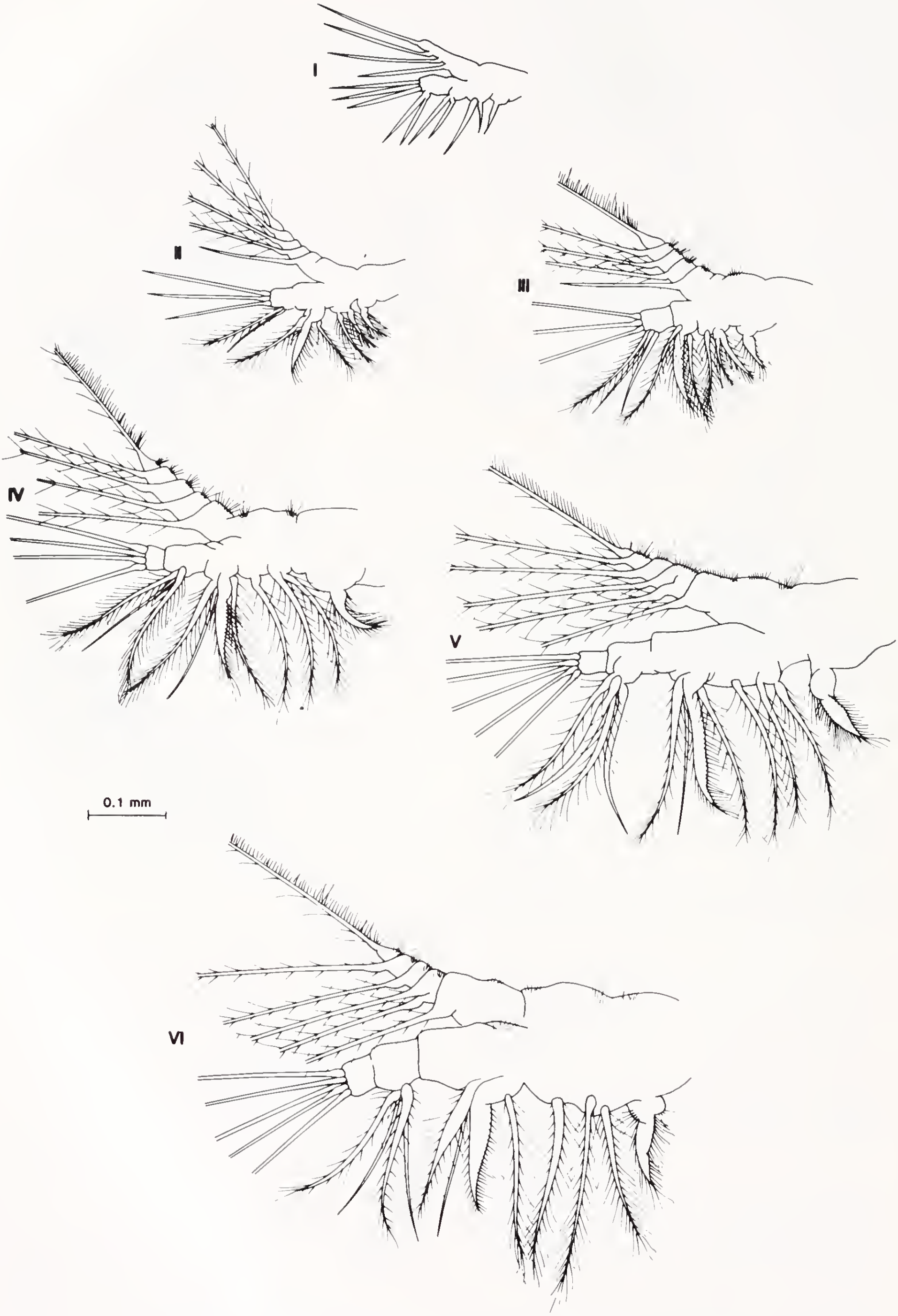
I
II
III
IV
V
0.1 mm
VI

FIGURE 6. *Lithotrya dorsalis.* Mandible of naupliar stages I–VI.

TABLE II

*Setation formulae for nauplii of* Lithotrya dorsalis

| Stage | Antennule | Antenna | Mandible |
|---|---|---|---|
| VI | S P P PSPS SP S PS S | 4P 8P PPSPP SSP FSFF PFSC G | P 5P 5S PSPS PSCP PPP G |
| V | S S P PSPS SP S P S | 3P 7PS PPSPS SSP FSPF PFSC G | P 5P 5S PSPS PSCP PPP G |
| IV | S P PSPS SP P S | 2P 7P PPSPS SSP F SF PFSC G | P 4P 4S PSP PSCP PPP G |
| III | S PSPS SP P S | 2P 5P PP P S P F F PPSC G | P 3PS 3S PSS P CP PPP G |
| II | SSPS SP P S | SP 4PS PS S S P F F SP C G | P 3PS 3S P S P CP PP G |
| I | SSSS SS S S | S 4S SS S S S S S SS G | S 3S 3S S S S S SS G |

arrangement of these carinae or ridges give the *L. dorsalis* cyprid cuticle a striated appearance quite distinct from the pitted appearance of the *Semibalanus balanoides* cyprid cuticle described by Walker and Lee (1976) and the somewhat hairy appearance of the *Lernaeodiscus porcellanae* (Ritchie and Høeg, 1981) and *Sacculina carcini* (Walker, 1985) cyprids due to the long setae present on their cuticles. The cuticular pores and setae of *L. dorsalis* and *S. balanoides* cyprids however, appear similar in shape and size. In *L. dorsalis,* pores (Fig. 9C) are round, small (approximately 5–8 $\mu$m) and numerous. Cuticular setae (Fig. 9B) are also numerous, particularly in the posterior region of the carapace. These setae extend from a pore-like process and vary slightly in length (5–8 $\mu$m). Dorsal and ventral marginal carinae sometimes continue uninterrupted for the entire length of the cyprid. Towards the mid-lateral section of the carapace (Fig. 9A) the carinae become shorter and appear to follow more closely the contour of the animal causing some degradation of their parallel arrangement. The distinct dorsal junction of the carapace valves including the dorsal hinge region is shown in high magnification (Fig. 8B).

## DISCUSSION

### *Rearing techniques*

Survivorship to the cyprid stage was relatively poor. Many permutations of diet, temperature, salinity, and light were tried with varying degrees of success. It was hoped that antibiotics could be avoided in the culture medium by lowering the tem-

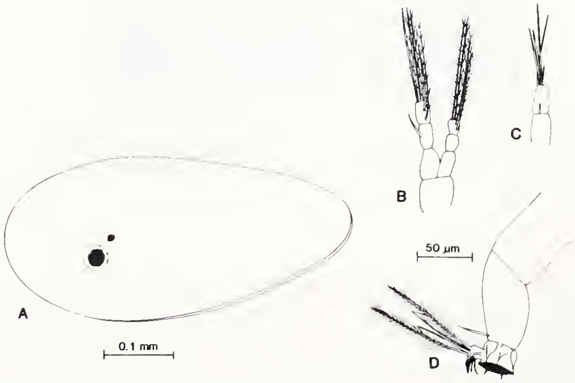

FIGURE 7. *Lithotrya dorsalis.* Carapace shield outline of cyprid (A); natatory appendage (B); caudal furca (C); and antennule (D).

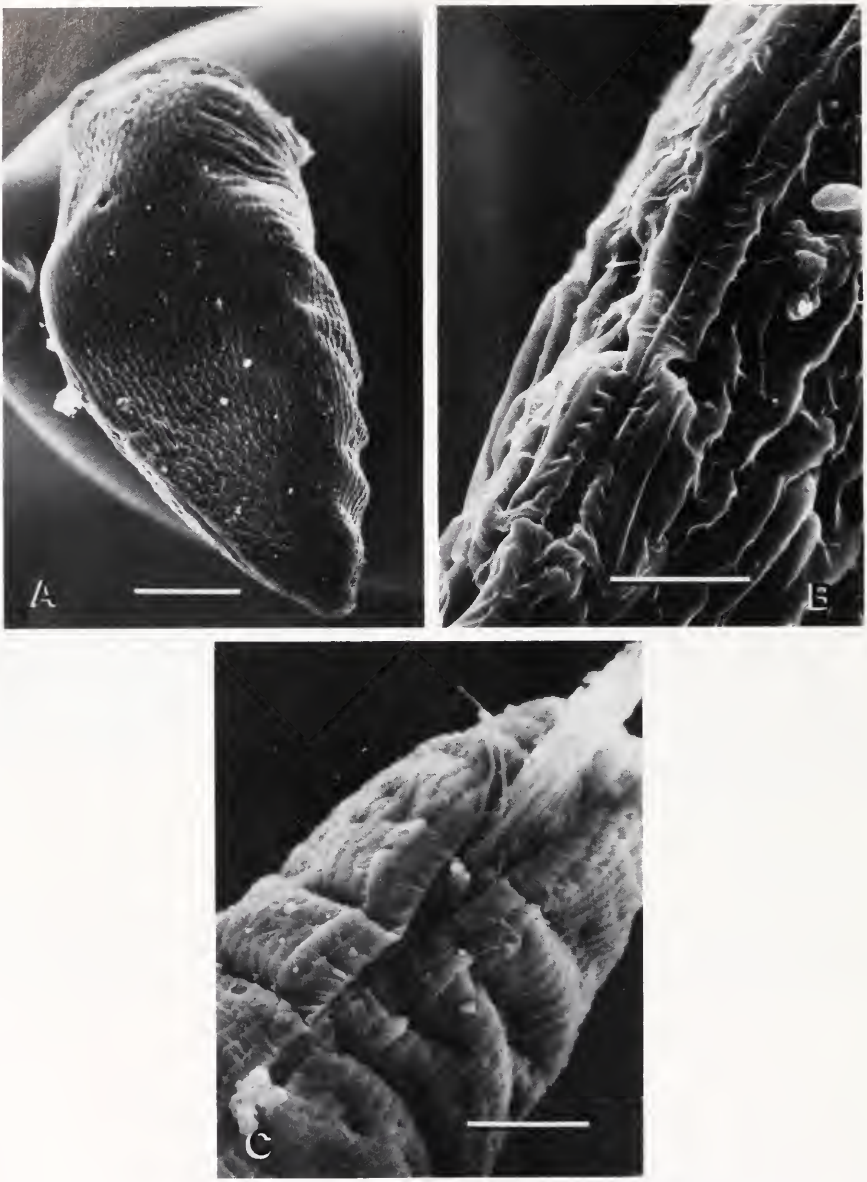

FIGURE 8. *Lithotrya dorsalis*. SEM Dorsal (A) and ventral (C) view of bivalved cyprid carapace; high magnification view of carapace junction including hinge region (B). (Scale bars: A = 50 μm; B = 10 μm; C = 100 μm).

perature from 30 to 22°C but larvae subsequently became sluggish and died off. Ambient seawater temperature when animals were sampled in September, 1983, was 32°C. This sluggish behavior may have resulted from thermal shock or perhaps a lower feeding efficiency at the decreased temperature (Scheltema and Williams, 1982). By lowering the salinity to 28 ppt in combination with antibiotics, entanglement of nauplii, particularly in mature cultures when appendage setation became

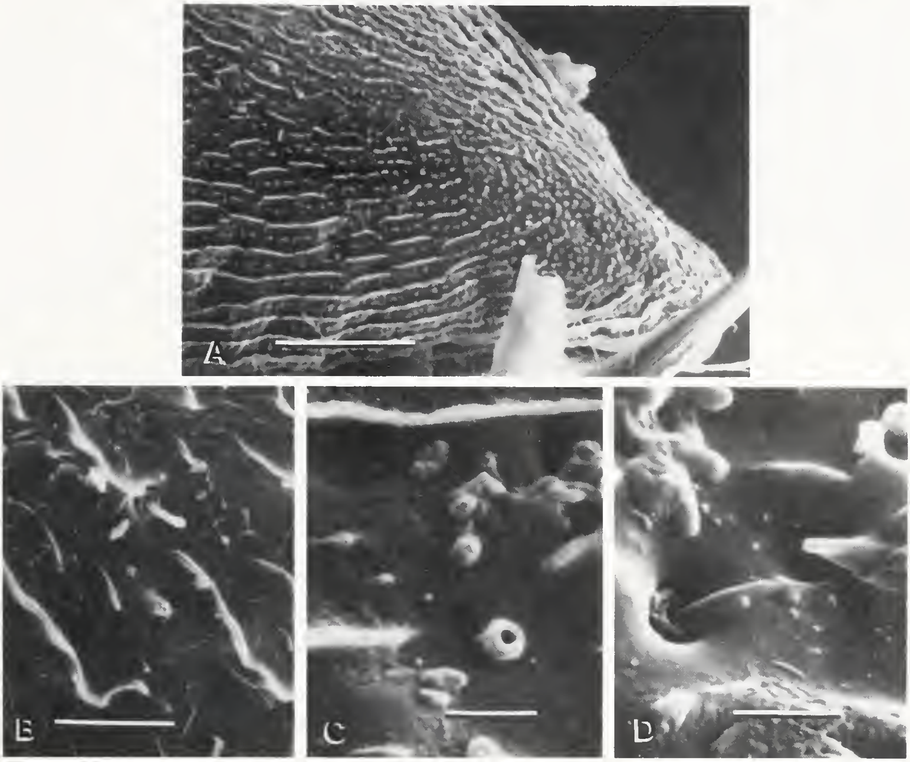

FIGURE 9. *Lithotrya dorsalis*. SEM Posterior lateral view of cyprid carapace (A) and cuticular structures: Seta (B), pore (C) and papillae (D). (Scale bars: A = 50 μm; B = 10 μm; C and D = 1.0 μm).

more elaborate, was avoided. In an attempt to increase the cyprid yield, while keeping the logistics of culture maintenance at a minimum, initial densities were increased from 1 or 2 to 6 or 8 nauplii per ml culture medium. Some of these high density cultures reached stage IV nauplius with no apparent deleterious effects. At this time, however, the algae being used as food (*Thalassiosira fluviatilus/Tetraselmis suecica*) became contaminated. By the time a new diet was selected (*Chaetoceros gracilis/Isochrysis* sp.), most cultures had been without food for 36 h. This apparent starvation resulted in substantial mortality, especially in older cultures. Younger cultures survived somewhat better, perhaps due to yolky material still present in these early naupliar stages. Because of the unexpected mortality, it was impossible to compare survivorship between low and high density cultures statistically. However, even though percent survival to the cyprid stage was lower in the "higher density" cultures, it was encouraging that actual cyprid yields in these cultures were higher than in the "low density" cultures.

Suspecting the possible release of chemical cues, several unsuccessful attempts to initiate metamorphosis of stage VI nauplius to the cyprid stage were made by placing stage VI nauplii in "conditioned" culture medium where metamorphosis had previously taken place.

The size of gravid individuals at the time of sampling (September and October) was small; their carinal-rostral axes averaged 3.4 mm. The larger individuals sampled at this time (carinal-rostral axes as large as 7.8 mm) were not gravid. This may have

TABLE III

*Measurements of scalpellid cyprid larvae*

| Species | Length (μm) | Width (μm) | Author |
|---|---|---|---|
| *Capitullum mitella* | 1050 | 400 | |
| *Calantica spinosa* | 850 | 385 | Yasugi, 1937 |
| *Scalpellum scalpellum* | 686 | 329 | Batham, 1946 |
| *Pollicipes polymerus* | 425 | 232 | Kaufmann, 1965 |
| *Lithotrya dorsalis* | 518 | 240 | Lewis, 1975 |
| | | | Dineen (present study) |

been a consequence of sampling at the end of the breeding season. Reproductive patterns in *Lithotrya dorsalis* have not been documented. Attempts to induce reproduction in the laboratory by increasing food availability and temperature failed.

### *Comparison of scalpellid larvae*

Adaptive radiation in the scalpelloids has given rise to the more advanced sessile barnacles [Suborders: Brachylepadomorpha (now extinct), Verrucomorpha, and Balanomorpha (Newman, 1982)]. It is noteworthy that the basic sequential pattern of larval development of this relatively primitive scalpellid barnacle is remarkably similar to that of other more advanced cirripeds described to date.

When comparing the larval stages of *Lithotrya dorsalis* with the four other described scalpellid species, the most striking similarity occurs between the cephalic shield outlines of *L. dorsalis* and *Capitullum mitella* (Yasugi, 1937). Although the length of *L. dorsalis* nauplii is greater at all stages, the same basic shield shape, along with the elongated and spinulated thoraco-abdominal process and dorsal thoracic spine and in particular, the elongated posterior shield spines in larval stages IV–VI, are present in both species. Rudimentary posterior shield spines appear in *C. mitella* naupliar stages II and III. Nauplii of the three other scalpelloids described, *Calantica spinosa* (Batham, 1946), *Scalpellum scalpellum* (Kaufman, 1965), and *Pollicipes polymerus* (Lewis, 1975) have relatively abbreviated thoraco-abdominal processes and dorsal thoracic spines and appear to lack posterior shield spines entirely. The unilobate labra of *L. dorsalis, P. polymerus,* and *C. mitella* are similar in outline, having a blunt posterior margin with numerous teeth, particularly in later stages. This same region in the naupliar labrum of stage VI *C. spinosa* appears narrowly rounded, with a single middle tooth. Labra of stage I nauplii of both *L. dorsalis* and *C. mitella* widen in the proximal region. Unfortunately, setation formulae for *C. mitella* are not given.

Cyprid sizes of these five scalpellids are compared in Table III.

The similarity between *Lithotrya dorsalis* and *Capitullum mitella* nauplii stimulate two questions. (1) Are these two species more closely aligned than presently thought? [Zevina (1981) places them in separate subfamilies: *Lithotryinae* and *Pollicipinae,* respectively). (2) More fundamentally, how reliable are naupliar characteristics as indicators of phylogenetic affinities among the Cirripedia?

## ACKNOWLEDGMENTS

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Reference: *Biol. Bull.* **172:** 299–306. (June, 1987)

# STUDIES OF THE ECDYSIOTROPIC ACTIVITY OF JUVENILE HORMONE IN PUPAE OF THE TOBACCO HORNWORM, *MANDUCA SEXTA*

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## ABSTRACT

Topical administration of a juvenile hormone analog (JHA) to pupae of the tobacco hornworm accelerated the initiation of adult development and proved to be an ecdysiotropic stimulus even in the absence of the brain. Nevertheless, individuals developing in response to JHA were never normal. In fact, doses of JHA too low to accelerate development often provoked typical abnormalities in the resulting moths. Allatectomy of either diapause-destined or non-diapause pupae failed to alter the time course of adult development. In line with this finding, corpora allata (CA) from both diapause-destined and non-diapause pupae were without activity in two juvenile hormone (JH) bioassays. These results suggest that JH is not involved in the normal initiation of adult development. Indeed, it must be absent for normal development to proceed. Evidently, the ecdysiotropic effect of exogenous JH in pupae reflects a sensitivity to JH retained from the larval period.

## INTRODUCTION

Increased secretion of ecdysone by the prothoracic glands (PG) is believed to constitute the proximate stimulus for the termination of pupal diapause and initiation of adult development. This augmented synthesis is believed to depend on the production of an ecdysiotropic hormone by the pupal brain, the so-called prothoracicotropic hormone (PTTH). Thus, in many well-documented instances, removal of the brain can significantly delay or even prevent the onset of adult development (for review, see Safranek and Williams, 1980).

In previous work from this laboratory we have examined the regulation of adult development in pupae of the tobacco hornworm, *Manduca sexta.* In these studies we have documented the critical role of the brain and PG in the normal initiation of adult development in both diapausing and non-diapausing pupae (Safranek and Williams, 1980). But in other studies our findings conflict with the classical model. For example, we have observed the initiation of adult development in brainless or decapitated pupae as well as in pupae lacking PG (Safranek and Williams, 1980; Safranek *et al.,* 1986). We have also demonstrated the ability of juvenile hormone (JH) or its analogs (JHA) to hasten the termination of pupal diapause and the initiation of adult development (Safranek *et al.,* 1980). These findings suggested that the endocrine system governing adult development in the hornworm might be significantly different from that defined by the classical model. In the present study we focus especially on

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Abbreviations: CA = corpora allata; JH = juvenile hormone; JHA = juvenile hormone analog; LD = long-day; SD = short-day; PG = prothoracic gland; PTTH = prothoracicotropic hormone; RIA = radioimmunoassay.

the ability of exogenous JH to accelerate the termination of pupal diapause and examine its possible role in the normal onset of adult development.

## MATERIALS AND METHODS

Hornworms were reared as described previously (Safranek and Williams, 1980) under either a short-day (SD, 12L:12D) photoperiod or a long-day (LD, 17L:7D) photoperiod at 25°C. The day of pupation is termed Day 1 of the pupal stage, and the first seven days of the pupal stage Week 1. Operations and ligations were performed as described previously (Safranek and Williams, 1980). Allatectomy was performed through a small horizontal incision in the vertex of the head within 12 h after pupal ecdysis. The corpora cardiaca were left *in situ* in this procedure. Sham operations on the CA were performed with complete visualization of these glands. In all experiments the initiation of development was recognized under the dissecting microscope by tracheal apolysis in the pupal wings.

The JHA "Hydroprene" (ZR-512, Zoecon Corp., technical grade) was used in certain experiments. For topical application, it was dissolved in acetone and dispensed onto the thorax with a 100 μl Hamilton syringe on a repeating dispenser.

The endocrine activity of individual CA was evaluated by the use of the "black larval assay" as described by Safranek and Riddiford (1975). JH secretion was also bioassayed by the implantation of CA into the heads of non-diapausing LD pupae within 24 h after pupal ecdysis: After the completion of development, the moths were scored for JH-induced morphological aberrations of the type described by Riddiford and Ajami (1973).

Ecdysteroid levels were measured by radioimmunoassay as previously described (Carrow *et al.*, 1981) using 20-OH-ecdysone as the standard.

## RESULTS

### *Effects of juvenile hormone application on diapausing pupae*

Groups of 50 diapause-destined SD pupae received one of three graded doses of JHA in a single topical application on the first day after pupal ecdysis. A control group of 50 similar pupae was treated with the acetone solvent only. The initiation of adult development was monitored at weekly intervals.

As shown in Figure 1, the two lower doses of JHA failed either to accelerate or to retard development over and beyond that of the controls. By contrast, about half of the individuals that received the highest dose of 200 μg terminated diapause prematurely; the rest, which were not distinguished by their sex, did not develop notably faster than the controls. Additional experiments demonstrated that *all* individuals initiated development within 3 weeks of pupation after a single administration of 400 μg (n = 20) or after daily applications of 20 μg over 7 days (n = 15).

Those pupae initiating development during the first 6 weeks in response to the high dose of 200 μg uniformly displayed pronounced JH-related abnormalities of the type previously described by Riddiford and Ajami (1973). These abnormalities were most reliably apparent in the compound eyes where the occurrence of facet-free crescents regularly provided clear evidence of an individual's exposure to JHA. Even when the dose was too low to accelerate the termination of diapause, as in the case of pupae receiving a single dose of 20 μg, those individuals which initiated development within the first 4 weeks after pupation showed typical JH-induced abnormalities, often limited to the compound eyes.

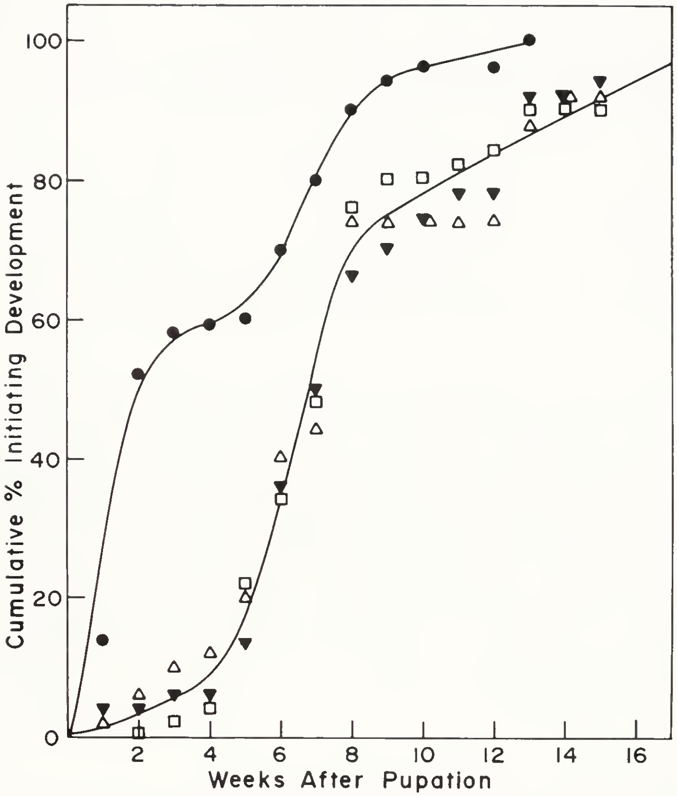

FIGURE 1. Initiation of development by groups of short day, diapause-destined pupae receiving topical application of JHA on the day of pupal ecdysis. Dosages of hormone per pupa were 200 $\mu$g (●), 20 $\mu$g (▼), 2 $\mu$g (□). Hormone was delivered in 2 $\mu$l acetone and one group of pupae received topical application of 2 $\mu$l acetone only (△). Pupae were examined for tracheal apolysis at weekly intervals. The cumulative percentage of each group demonstrating apolysis is plotted as a function of time after pupation. The lines are drawn by inspection.

*Ecdysiotropic effects of JHA*

We next inquired whether the acceleration of development witnessed in preparations treated with JHA was accompanied by an elevation of the ecdysteroid titer. Three groups were established: intact diapause-destined pupae, sham-operated diapause-destined pupae, and diapause-destined pupae from which the brain had been extirpated on Day 2. To individuals in each of these groups we administered daily topical applications of 20 $\mu$g of JHA beginning on pupal Day 3 and continuing until the onset of adult development. Hemolymph was collected on the day that tracheal apolysis was first observed and assayed for ecdysteroids by RIA. Ecdysteroid levels were also measured in hemolymph from two JHA-free control groups, one consisting of 4-week-old diapausing pupae, the other of individuals that had been in diapause for at least 4 weeks and had undergone tracheal apolysis during the 24 h preceding hemolymph collection.

Intact JHA-treated pupae initiated development 5–14 days after initiation of

TABLE I

*Effects of JHA administration on the ecdysteroid titer at the outset of adult development*

| Procedure | Stage | Number | Ecdysteroid titer* |
|---|---|---|---|
| SD pupa, intact | Pupal diapause, 4 weeks | 12 | 0.23 ± 0.05 |
| SD pupa, intact | Tracheal apolysis | 12 | 19 ± 7 |
| SD pupa, intact + JHA | Tracheal apolysis | 12 | 43 ± 12 |
| SD pupa, sham + JHA | Tracheal apolysis | 5 | 49 ± 15 |
| SD pupa, − brain + JHA | Tracheal apolysis | 10 | 30 ± 15 |

* The ecdysteroid titer is expressed in μg/ml 20-OH-ecdysone equivalents.

treatment, 50% by the seventh day. Sham-operated individuals developed 7–18 days after initiation of treatment, 50% by the ninth day. All brainless individuals developed 5–35 days after initiation of treatment, 50% by the eleventh day. The ecdysteroid titers of these groups and of the control pupae are shown in Table I. Manifestly the JHA treatment had a marked ecdysiotropic effect on all individuals. The average ecdysteroid titer achieved at the outset of development was two orders of magnitude higher than that typical of diapausing pupae. Moreover, the ecdysteroid levels noted in these groups were higher even than those typically attained by intact untreated pupae at a similar developmental stage. This was true even of brainless JHA-treated pupae. No correlation was seen between the ecdysteroid titer and the day of treatment on which development was first noted.

### *Effects of allatectomy*

The CA were removed from 50 diapause-destined SD pupae 6–12 h after pupal ecdysis. Another 50 were sham-operated, while an additional 100 served as unoperated controls. As shown in Figure 2, all three groups underwent a typical and essentially identical diapause. Once apolysis was initiated, the individuals of all three groups developed into morphologically normal adults in the usual period of 3 weeks. A duplicate experiment on three groups of 25 pupae provided substantially the same results.

The experiment was repeated on three groups of potentially non-diapausing LD pupae. The results are summarized in Figure 3. Unoperated controls initiated development in an average of 4.1 days, sham-operated controls in 5.5 days, and allatectomized pupae in 6.6 days. The one day delay of allatectomized pupae relative to controls could have been due to the slightly greater injury involved in the allatectomy. It could also reflect the putative role of the CA as neurohemal organs in this species (Nijhout, 1975; Gibbs and Riddiford, 1977; Agui *et al.*, 1980; Carrow *et al.*, 1981).

### *Assay of the JH activity of pupal corpora allata*

Secretion of JH by pupal CA was assayed in *bl* larvae and in non-diapausing pupae as described in Materials and Methods. To this end, groups of 12 non-diapausing pupae received CA from Day 1 or Day 3 non-diapausing pupae or from Day 1 or Day 14 diapause-destined pupae. Each assay pupa received one pair of CA. All individuals subsequently developed into morphologically normal adults—a result documenting the inactivity of the pupal CA. By contrast, implantation of only a

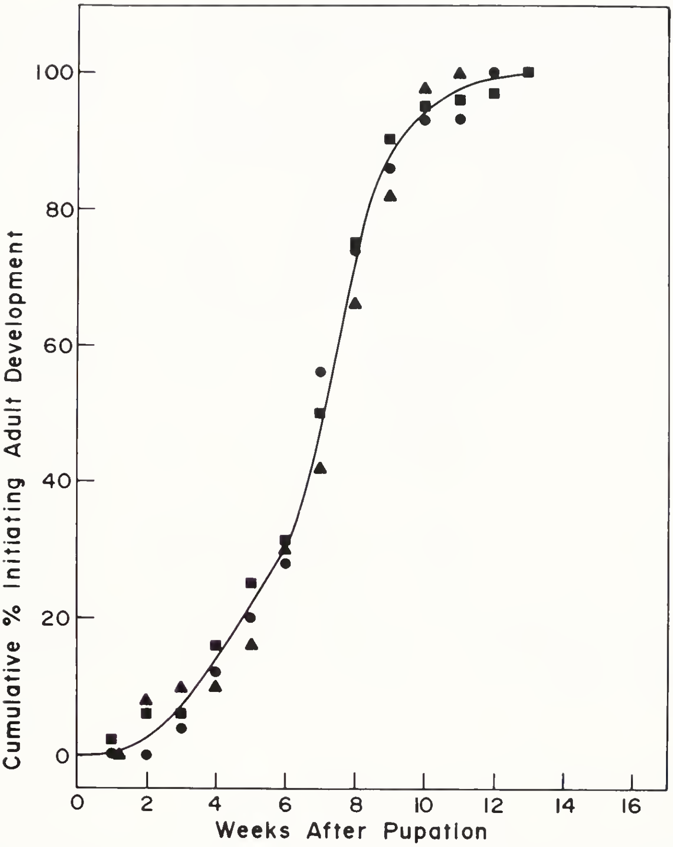

FIGURE 2. Lack of effect of allatectomy on the termination of pupal diapause. Diapause-destined (SD) pupae were operated 6–12 h after pupal ecdysis and were examined for initiation of development at weekly intervals. The three groups consisted of 50 allatectomized pupae (■), 50 sham-operated pupae (●), and 100 unoperated controls (▲). The cumulative percentage of each group showing apolysis is plotted as a function of time after pupation. The line is drawn by inspection.

single CA from a Day 1 fifth-instar LD larva into each of 12 additional non-diapausing pupae produced a range of typical JH-related aberrations in all resulting adults.

In additional tests, individual CA from the same classes of donors as above were implanted for bioassay into appropriate *bl* larvae. The average score of each group of 12 pupal CA was less than 0.5, indicating essentially no detectable activity. By contrast, the CA from a group of 12 early fifth-instar larvae averaged an essentially maximal score of 2.8. Thus, whereas CA from larvae at the outset of the fifth instar were highly active in both assays, none of the pupal CA possessed any detectable JH activity when similarly tested.

## DISCUSSION

The foregoing findings leave little doubt that elevation of the ecdysteroid titer is the ultimate cause of the onset of adult development in pupae subjected to juvenoid

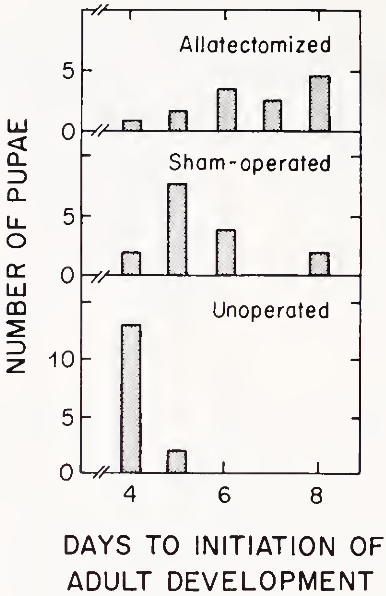

FIGURE 3. Effect of allatectomy on the initiation of development by non-diapausing (LD) pupae. Operations were performed 6–12 h after pupal ecdysis. Individuals were examined daily for tracheal apolysis. Each of the three groups comprised 15–18 pupae.

exposure, just as in the case of untreated pupae. Thus, as illustrated in Table I, the ecdysteroid titer in JHA-treated preparations at the outset of adult development achieved average levels 100 times that typical of untreated diapausing pupae, and in fact exceeded that encountered in untreated individuals spontaneously initiating adult development. Since this same result was observed in brainless pupae, the brain was manifestly unnecessary for mobilizing ecdysone sufficient to provoke the developmental response. Moreover, since the ecdysteroid titer at the outset of JHA-provoked development was actually greater than that witnessed normally, the acceleration of development occurring after JHA administration cannot be attributed to an enhanced tissue sensitivity to ecdysteroids, as has been suggested (Sehnal *et al.*, 1981).

The most parsimonious hypothesis is that the administered juvenoid acted directly on the PG to provoke the secretion of ecdysone. Nevertheless, this possibility has been questioned in the literature. Thus, the suggestion has been made that JH acts indirectly by mobilizing a diffusable factor from the fat body which in turn enhances ecdysone secretion by the PG; a direct action on the PG has been specifically denied (Gruetzmacher *et al.*, 1984). Other experiments have been interpreted to indicate an effect of JH on PG through an indirect mechanism requiring the PG to be *in situ* (Sehnal *et al.*, 1981). The question of direct or indirect action of JH on the PG remains, in our opinion, unresolved; we shall consider it in further detail in a subsequent communication.

Though JH can accelerate the initiation of adult development of diapausing *Manduca* pupae and has been shown to have pronounced ecdysiotropic effects at this stage, the hormone appears to play no role in normal adult development or in the maintenance or termination of diapause. Indeed, JH must be absent for normal development to proceed. Thus as we have seen, the initiation of adult development could not be stimulated by JHA without also producing morphological aberrations in the resultant adults. Indeed, abnormalities could be brought about by doses of JH that were too low to accelerate development. In addition, as summarized in Figures 2 and 3, allatectomy produced virtually no delay in the development of either diapausing or non-diapausing pupae. Finally, pupal CA proved to be completely inactive in two different bioassays for JH. In conjunction with our previous results (Safranek and Williams, 1980) these findings make clear that the pupal brain's ecdysiotropic effects are not mediated through activation of JH production by the pupal CA.

The present findings confirm and extend previous observations of Bradfield and Denlinger (1980) that allatectomy failed to alter the duration of the *Manduca* pupal diapause. Moreover, in that same study JH could not be detected in the hemolymph derived from diapausing pupae of several different ages—a finding consistent with the inactivity of pupal CA documented in the present investigation. The current results also agree with prior findings documenting the lack of a role for JH in the initiation of adult development in silkworm pupae (Ichikawa and Nishiitsutsuji-Uwo, 1959; Williams, 1959, 1961; Gilbert and Schneiderman, 1959).

One remaining question concerns the *raison d'etre* for the marked sensitivity to juvenoids during the pupal stage. Though the application of JHA can accelerate the termination of pupal diapause, the aberrant development induced by even low levels of JH makes clear that at least the early phases of adult morphogenesis require a JH-free environment. The pupal epidermis has long been known to retain from the larval stage the ability to respond to exogenous JH at the outset of adult development. Similarly the ecdysiotropic effects of JH administered during the pupal stage may reflect a normally unused sensitivity carried over from the larval period when JH is typically present and may have significant effects on ecdysone secretion.

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# IMMOBILIZATION OF THE PREDATORY GASTROPOD, *NUCELLA LAPILLUS*, BY ITS PREY, *MYTILUS EDULIS*

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## ABSTRACT

Slow moving and sessile organisms cannot escape predators and must rely on other defenses. Laboratory experiments show that mussels (*Mytilus edulis*) immobilize predatory gastropods with byssal threads. This study assesses the risk of immobilization faced by the gastropod, *Nucella lapillus*, under natural conditions. Nearly 30% of the *N. lapillus* within mussel beds are trapped, and the per day risk to a predator in a mussel bed is between 0.010 and 0.038. The mussels nearest to the predator attach 20 or more byssi to the body whorl of its predator's shell. Byssi are retracted, flipping the predator over. *Nucella lapillus* are rarely found in mussel beds (0.10 individuals per 0.25 $m^2$ in beds and 2.38 per 0.25 $m^2$ out of beds). It is not clear if *N. lapillus* are avoiding mussel beds for other reasons, such as exposure to predators, or are seeking alternative prey which do not co-occur with mussels. Nevertheless, mussels pose a danger to predatory gastropods.

## INTRODUCTION

Predation poses one of the greatest risks for slow-moving and sessile organisms and often limits the distribution of many such species (Connell, 1961; Paine, 1974; Menge, 1976; Vermeij, 1978; Peterson, 1979). While some clonal sessile marine invertebrates can survive partial destruction by predators (*e.g.* Harvell, 1984), nonclonal individuals must inhibit access by predators or live in areas where predators are otherwise excluded (Schmitt, 1981; Moran, 1985; Warren, 1985).

*Mytilus* is one of the most successful and ubiquitous genera on rocky shores and forms extensive beds that appear to be completely defenseless against predators. Other shelled marine invertebrates co-exist with predators by virtue of reinforced shells (Vermeij, 1978) or have distasteful tissue (Thayer, 1985), yet *M. edulis* (the common blue mussel) has neither defense. Paradoxically, *M. edulis* flourishes in association with predators, and in sheltered bays in New England, mussels occur with a common predatory gastropod, the dogwhelk *Nucella lapillus.* Dogwhelks readily take mussels and can easily kill them by drilling through the mussel's relatively thin shell (Menge, 1978a, b, 1983; Hughes and Dunkin, 1984).

This study assesses the effectiveness of a novel antipredator defense of *Mytilus edulis* against *Nucella lapillus.* Groups of mussels immobilize dogwhelks with byssal threads, which are secreted by a gland in the mussel's foot and are normally used by the mussel to attach to the surface. This defense by *M. edulis* against predators has been observed on the east coast in response to the presence of the oyster drill *Urosalpinx cinerea* (Carriker, 1981) and on the west coast of North America in response to attacks by *Nucella emarginata* and *N. lamellosa* (Wayne, 1980). Similar responses by

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*M. californianus* to *N. canaliculata* have been observed on the west coast of North America (T. H. Suchanek, pers. comm.).

Studies with *Mytilus edulis* have been done only in the laboratory. Carriker's (1981) observations were made at 15°C, and *U. cinerea* were inactive at this temperature. Wayne (1980) has documented a series of specific responses by mussels to whelks. Besides attachment of byssal threads, Wayne observed a mussel would open and close its valves and would extend its mantle. The responses were highly stereotyped and occurred only in the presence of *N. lamellosa* and *N. emarginata*. The whelks were assumed to be at risk because they used their radulae to strike at the mussel's foot during attempts to attach a byssus (Wayne, 1980).

However, there has been no assessment of the risk to individual whelks. This report documents the distribution of *N. lapillus* with respect to *M. edulis* and the risk of being trapped by byssal threads under natural conditions.

## MATERIALS AND METHODS

Observations were made between June 1983 and July 1986 just below Mean Low Water (−0.3 to 0.0 MLW) at five sites within protected bays on Swans Island, Maine (44° 10′ N, 68° 25′ W). Four sites (Long Cove, Mill Pond, Mill Pond South, and Mill Pond Point) on the eastern shore of Burnt Coat Harbor, and a fifth site on the eastern shore of Mackerel Cove were used. Long Cove was used only in 1983. Mill Pond South and Mill Pond Point sites were less than 200 m apart; the other sites in Burnt Coat Harbor were 500–1000 m apart. The substratum at all sites was a mixture of granite outcrops, boulders, and cobbles. Barnacles (*Semibalanus balanoides*) and mussels (*Mytilus edulis*) were the most common sessile organisms, and algae covered less than 5% of the surface (see Petraitis, 1987, for a more detailed description of sites).

To document the distribution of *Nucella lapillus* in relation to mussel beds, the amount of surface covered by mussels and the abundance of *N. lapillus* inside and outside mussel beds was estimated. In June 1986, transect lines (88–125 m long) were run parallel to the shore, and the species present were noted at 1 m intervals. On 23–24 June 1986, abundance of *N. lapillus* was sampled with 50 × 50 cm quadrats which were placed outside or inside mussel beds at each site. Mill Pond Point was also sampled on 3 June 1984. Data from the quadrats were bimodal, so Kruskal-Wallis tests (Sokal and Rohlf, 1981), which were corrected for ties, were used to test for differences.

*Nucella lapillus* that were trapped within mussel beds were tallied over a four year period. At low tide, mussel beds were searched for dogwhelks, and each live dogwhelk was scored as trapped or free. Dogwhelks seen on the margins of the mussel beds were also counted, and some of these individuals were checked for evidence of byssi. No site was surveyed more than once a year.

Two manipulations estimated the rate at which dogwhelks were trapped. In the first experiment, dogwhelks were caged in 15 × 45 × 50 cm baskets for 3 weeks with mussels covering the bottom of the basket. Baskets were made of stainless steel mesh with openings of about 5 × 5 mm. Clumps of mussels were carefully transferred into the cages three to five days before the start of the experiment. On 16 July 1983, three baskets with 25 dogwhelks per basket were started at Long Cove. Between 22 and 23 June 1986, four baskets with 20 dogwhelks per baskets were started at Mackerel Cove, Mill Pond, and Mill Pond Point. Additional treatments with 20 dogwhelk shells per basket or 20 *Littorina littorea* (a herbivorous gastropod commonly found in mussel beds) per basket were used in 1986 to check if mussels were preferentially trapping

TABLE I

*Mean number of* Nucella lapillus *per 0.25 m² in and out of mussel beds*

| | Sampling | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Within mussel beds | | | | Outside mussel beds | | | | |
| Location | Mean | S.D. | n | Prop. | Mean | S.D. | n | Prop. | T |
| Mill Pond South | 0.00 | 0.00 | 20 | 1.00 | 0.15 | 0.49 | 20 | 0.90 | 2.05 |
| Mill Pond Pt. 84 | 0.23 | 0.51 | 26 | 0.81 | 6.03 | 7.36 | 29 | 0.17 | 25.58 |
| Mill Pond Pt. 86 | 0.15 | 0.49 | 20 | 0.90 | 3.26 | 4.19 | 19 | 0.32 | 14.38 |
| Mill Pond | 0.05 | 0.22 | 20 | 0.95 | 1.90 | 3.57 | 20 | 0.55 | 8.84 |
| Mackerel Cove | 0.05 | 0.22 | 20 | 0.95 | 0.57 | 0.75 | 21 | 0.57 | 7.93 |

n gives the number of quadrats, and prop. is the proportion of quadrats without *N. lapillus*. Column T shows test values for Kruskal-Wallis tests.

live *N. lapillus*. Three baskets of these additional treatments were set out at each site. Data from 1986 were analyzed as a two-way analysis of variance of treatments by sites. The three sites served as blocks, and the analysis was a mixed model with replication. The data, proportions of individuals trapped per cage, were arcsine transformed for the analysis (Johnson and Klotz's transformation, see Sokal and Rohlf, 1981).

The second manipulation was a mark-recapture study. Dogwhelks were marked with red paint and placed in the middle of a mussel bed. On 2 June 1984, 100 marked dogwhelks were released at Mackerel Cove, and individuals were recovered 4 days later. On 24 June 1986, 238 individuals were marked and half were released at Mackerel Cove and half at Mill Pond Point. Recaptures were done three days later. At the same time in 1986, 68 marked empty *Nucella* shells were set in mussel beds at Mackerel Cove and Mill Pond Point to assess the rate at which empty shells were normally trapped and incorporated into mussel beds.

## RESULTS

*Nucella lapillus* were rare in mussel beds, even though mussels were usually the most common sessile organism (Table I, Fig. 1). The overall average density of dogwhelks within mussel beds was 0.10 per 0.25 $m^2$ while the density outside beds was 2.38 per 0.25 $m^2$. For each site except Mill Pond South, the difference was significant (Table I). In total, dogwhelks were found in only 12 of the 106 quadrats which were within beds, but in 58 of the 109 quadrats outside beds. The density outside beds is within the range of densities found at these sites at other times (Petraitis, 1987) but below the range of densities reported for more exposed locations (Lubchenco and Menge, 1978).

Just over 27 percent of all live dogwhelks found within beds were tightly bound with byssal threads (average of entries in Table II). Over the four year period, only 293 individuals were found within beds, although 4186 dogwhelks were recorded on the margins of mussel beds. Although each site was searched as nearly as possible with the same amount of effort, the number of dogwhelks seen and trapped varied year to year and site to site (Table II).

Position and number of the byssal threads on the trapped *Nucella lapillus* were very distinctive. The three or four mussels closest to, but not necessarily touching,

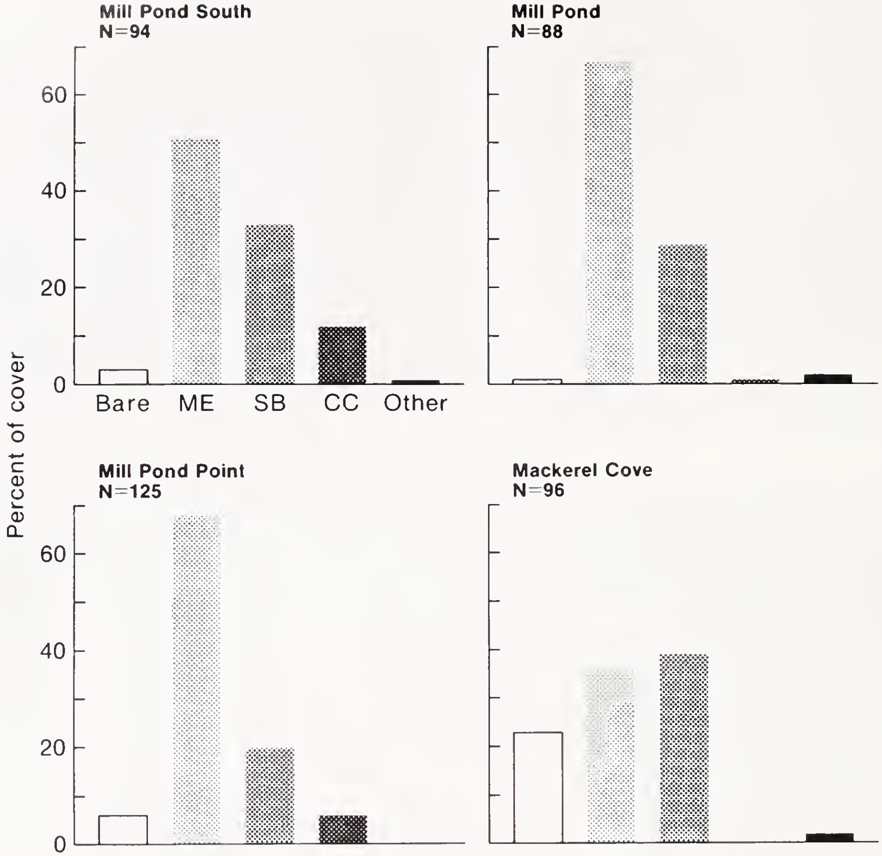

FIGURE 1. Summary of transect data. Abbreviations for species are: ME for *Mytilus edulis*, SB for *Semibalanus balanoides*, CC for *Chondrus crispus*, and OTHER for other algae. N is the number of points sampled.

the dogwhelk attached 20 or more byssal threads to the body whorl of the dogwhelk. The threads appeared to be retracted, flipping the dogwhelk over so its foot did not touch the surface. Dogwhelks were often seen extending the foot in an attempt to right and free itself, although the inverted position and the collective strength of the threads made escape seem unlikely.

Outside mussel beds, the proportion of dogwhelks with remains of byssal threads was highly variable but indicated many encounters between dogwhelks and mussels. From lowest to highest, the proportion of dogwhelks with byssi: 0.05 at Mackerel Cove (n = 137), 0.40 at Mill Pond Point (n = 311), 0.52 at Mill Pond South (n = 189), and 0.75 at Mill Pond (n = 107).

There were significant effects of treatment and site in the analysis of variance of the 1986 caging data (Fig. 2). However, only 3% of the total variance was due to differences among the three sites (see Sokal and Rohlf, 1981, for estimation of variance components), and byssal threads were attached more often to live *Nucella lapillus* and empty shells of *N. lapillus* than to *Littorina littorea*. Empty shells were very frequently trapped, but these shells were normally found near the bottom of the mus-

TABLE II

*Proportion of live* Nucella lapillus *trapped by byssal threads of mussels*

| | Dates of sampling | | | |
|---|---|---|---|---|
| Location | 9–14 Jul 1983 | 4–6 Jun 1984 | 2–5 Jul 1985 | 18–26 Jun 1986 |
| Long Cove | 0.42<br>(ND) | ND | ND | ND |
| Mill Pond S. and Pt. | ND | 0.24<br>(29/842) | 0.07<br>(14/229) | 0.52<br>(64/564) |
| Mill Pond | 0.42<br>(12/62) | 0.16<br>(25/955) | 0.36<br>(11/491) | 0.71<br>(17/177) |
| Mackerel Cove | 0.00<br>(47/97) | 0.15<br>(39/149) | 0.15<br>(40/151) | 0.25<br>(8/411) |

ND stands for no data. Ratios in parentheses are the total number of live dogwhelks (trapped and free) found in mussel beds over the total number seen in and out of beds while sampling. The proportion is based on the number found in the mussel bed, *e.g.*, for Mill Pond in 1983, 0.42 = 5/12.

sel clump and were attached with very few byssi. In contrast, the live *N. lapillus* which were trapped were usually on the top of the mussel clump and covered with many byssal threads. The low incidence of trapping *L. littorea* may be unrelated to the ability of mussels to distinguish between dogwhelks and periwinkles. Periwinkles are much more mobile than dogwhelks, and thus periwinkles may not be sitting in one spot long enough for a mussel to attach a byssus.

Based on the caging study, the overall per day risk was 0.014 with a quarter of the dogwhelks trapped after three weeks. A dogwhelk had a half-life of 50 days, and an individual had less than a 1 in 5 chance of surviving its active season if it remained within a bed during summer and early fall (about 120 days).

The average per day risk was 0.038 (S.D. = 0.020) for the *Nucella lapillus* in the mark-recapture study (Table III). Dogwhelks were trapped very quickly, and stood a 50:50 chance of being trapped in 2 to 4 weeks. Empty shells of dogwhelks which were marked and placed in mussel beds were also trapped but at a lower rate (Table III). For live *N. lapillus*, the estimates may be high because it was assumed that all unaccounted dogwhelks left the mussel bed. If these individuals remained in the bed but were undiscovered, then the average risk was reduced to 0.010 per day.

Success at finding marked shells and dogwhelks did not differ; in 1986, 75.0% of the empty shells and 70.6% of *N. lapillus* were recovered. A two by two Chi-square test of independence, using data from 1986 in Table III, was not significant ($\chi^2 = 0.85$ with one degree of freedom).

## Discussion

Observations and manipulations show that *Nucella lapillus* runs a substantial risk of being immobilized by byssal threads of *Mytilus edulis*. Given the level of risk, one would expect dogwhelks to avoid mussel beds even though mussels are a common prey item of dogwhelks (Menge, 1976; Hughes and Dunkin, 1984). Although sampling confirms this notion (Table I), it is possible that *N. lapillus* are not so much avoiding mussel beds but are searching for an alternative prey, such as the barnacle *Semibalanus balanoides*, outside of the mussel beds. It is also possible that predators

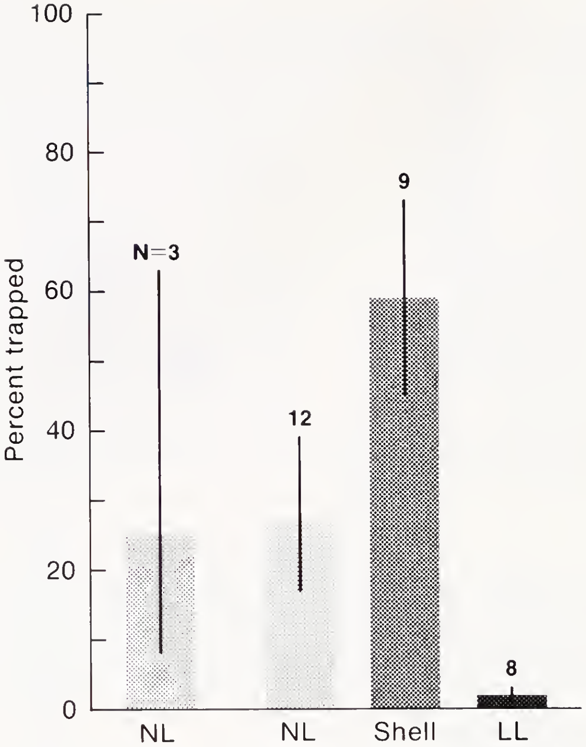

FIGURE 2. Means ± 95% confidence limits with sample sizes (N) for percentage of individuals trapped by byssi in the caging experiments. The leftmost bar gives 1984 data; the rest for data collected in 1986. The treatments are abbreviated as: NL for *Nucella lapillus*, LL for *Littorina littorea*, and SHELL for shells of *Nucella lapillus*. Blocks were ignored in calculating the means and confidence limits for 1986 data; one observation is missing from the LL treatment. Based on analysis of variance of 1986 data, F = 26.77 for the effect of treatments (tested over interaction mean square which is 134.58 and has 4 degrees of freedom) and F = 4.44 for the effect of sites (tested over error mean square which is 60.37 and has 20 degrees of freedom). Main effects are significant at the 5% level, and there is no significant interaction.

quickly remove *N. lapillus* from mussel beds or that mussel beds are an unsuitable habitat for other reasons.

Nevertheless, the rarity of *Nucella lapillus* in mussel beds is striking because mussels cover more than 50% of the surface at all sites except Mackerel Cove (Fig. 1). If the proportion of *N. lapillus* that are outside mussel beds is adjusted for the area covered by mussels, then less than 10% of all dogwhelks are found within mussel beds. For example, at Mackerel Cove 36% of the surface is covered by mussels, and there are 0.05 dogwhelks per 0.25 $m^2$ within beds and 0.57 dogwhelks per 0.25 $m^2$ outside mussel beds (see Table I and Fig. 1 for data). Adjusting the densities for the proportion of area occupied by mussels, this means 95% of the dogwhelks are not in mussel beds (*i.e.*, $0.95 = (0.64)(0.57)/[(0.64)(0.57) + (0.36)(0.05)]$).

The behavior of *Nucella lapillus* suggests large individuals of *Mytilus edulis* are dangerous prey. *Nucella lapillus* that have previously taken mussels drill well away from the mussel's foot and byssi, slightly ventral and anterior to the posterior adductor muscle (Hughes and Dunkin, 1984). While Hughes and Dunkin (1984) suggest this position gives the highest caloric return, it is also likely to be the least dangerous.

TABLE III

*Mark and recaptures of live individuals and shells of* Nucella lapillus

| Location | Marked |  | Recaptures |  |  | Risk (#/day) |
|---|---|---|---|---|---|---|
|  | Object | n | Trapped | Free | Not in bed |  |
| Mackerel Cove 1984 | Snails | 100 | 4 | 43 | 4 | 0.022 |
| Mackerel Cove 1986 | Snails | 119 | 10 | 50 | 29 | 0.061 |
|  | Shells | 68 | 0 | 49 | 0 | 0.000 |
| Mill Pond Pt. 1986 | Snails | 119 | 3 | 31 | 45 | 0.031 |
|  | Shells | 68 | 3 | 50 | 0 | 0.019 |

Column labeled "Not in bed" gives the number found on the margins and outside of the mussel beds. Risk is the exponential rate of trapping.

In addition, these "experienced" *Nucella lapillus* generally prey upon smaller mussels even though they do not provide the highest caloric return (Hughes and Dunkin, 1984), possibly because a small mussel cannot ward off an attack and thus involves less risk.

Cooperative defense by group-living animals is well-known (*e.g.*, Alexander, 1974; Buss, 1981), but its possible importance for sessile non-clonal organisms such as *Mytilus edulis* is not appreciated. Because *Nucella lapillus* is usually immobilized by several mussels, solitary mussels may be much more likely to be taken by *N. lapillus.* Thus mussel beds may provide a common, group protection and may explain the absence of solitary mussels in areas where dogwhelks are common. There are other possibilities. For example, mussels are also the prey of seastars, crabs, fishes, and shore birds (Mason, 1972; Menge, 1976; Edwards *et al.*, 1982; Hughes and Dunkin, 1984; Thayer, 1985), and it may be easier for these predators to remove a solitary mussel rather than one in the midst of a bed.

The behaviors of *Nucella lapillus* and *Mytilus edulis* and the success of *M. edulis* in immobilizing *N. lapillus* suggest byssal threads provide a specific and successful defense against predatory gastropods. It is possible that the mussel's response to predators is an elaboration of a cleaning behavior; *M. edulis* sweeps its shell with its foot preventing fouling by other organisms (Thiesen, 1972). However, the mussel's specific responses to *Nucella* (Wayne, 1980) and the mussel's characteristic placement of byssi on a dogwhelk's shell suggest this is not simply a generalized cleaning behavior. Moreover, the avoidance of mussel beds by dogwhelks (Table I), the use by a dogwhelk of its radula to strike at a mussel's foot (Wayne, 1980), and the positioning of dogwhelks on the valves of mussels (Hughes and Dunkin, 1984) support the notion that the mussel's behavior is a successful defensive action.

While the behavior of *Mytilus edulis* may be adapted as a specific defense against *Nucella,* it is possible other species of mussels could respond to predatory gastropods in the same way. Furthermore, if these behaviors are common, then the absence of a thick, reinforced shells may be a poor indicator of vulnerability to predation.

## ACKNOWLEDGMENTS

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# A DEUTEROSTOME-LIKE NEPHRIDIUM IN THE MITRARIA LARVA OF *OWENIA FUSIFORMIS* (POLYCHAETA, ANNELIDA)

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## ABSTRACT

The mitraria larva of *O. fusiformis* possesses a pair of nephridia which are not typical polychaete larval protonephridia, rather they resemble the pore canal-hydropore complex of deuterostome larvae. An ultrastructural analysis of the nephridium reveals a small body cavity, lined by monociliated podocytes, which opens to the exterior by a monociliated nephridioduct. Extracellular matrix between podocyte pedicels forms the filtration surface and podocyte and nephridioduct cilia produce the pressure gradient driving filtration. Therefore, each nephridium is considered a giant protonephridium. Although this organization is unique to *Owenia* among the annelids, it is identical ultrastructurally to the hydropore, pore canal, and adjoining coelom of deuterostome larvae, which is also a nephridium. It is concluded that this cytological similarity between the nephridia of the mitraria and deuterostome larvae reflects a cellular homology which has a wide distribution and is of limited usefulness in the recognition of relationships. The anatomical differences between them suggest they are, at best, serially homologous organs. Mitraria nephridia compare more favorably to the typical protonephridia (head kidneys) of polychaete trochophores and larval protonephridia of phoronids. Data support the view that *Owenia* is primitive among the polychaetes and suggest that the mitraria nephridium represents the plesiomorphic design of protonephridium within the Polychaeta.

## INTRODUCTION

A recent study testing the generality of a model explaining nephridial diversity in the Metazoa (see Ruppert and Smith, 1985; in prep.) reported that the pore canal-hydropore complex in bipinnaria larvae of echinoderms and tornaria larvae of hemichordates are functional nephridia (Ruppert and Balser, 1986). Our attention was subsequently drawn to the larval nephridium of the polychaete *Owenia fusiformis* by Wilson's (1932) classic study on the mitraria larva. His data indicate that the nephridium is not a typical polychaete larval protonephridium: monociliated or multiciliated terminal cells joined to a nephridioduct which opens on the larval hyposphere (Pemerl, 1965; Holborow, 1971; Wessing and Polenz, 1974; Heimler, 1981; Smith and Ruppert, in press). Instead, each nephridioduct joins a small sac-like cavity that attaches to the episphere by a muscle band. These nephridia resemble the pore canal-hydropore complex of deuterostome larvae.

Two theories are used to explain the evolution of the annelids. One proposes that the segmental coelom evolved directly from the acoelomate organization (Goodrich, 1945; Clark, 1964) and predicts that primitive characters will be shared by annelids

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and species of acoelomate taxa. The other proposes that the segmental coelom evolved from coelomate organization (Remane, 1963, 1967; Siewing, 1976, 1980, 1981) and predicts that primitive characters will be shared uniquely by annelids and species of coelomate taxa. The explanatory value of either theory depends on the recognition of homologous structures between annelids and other phyla of animals.

The organization of *Owenia fusiformis* has figured prominently in recent discussions of the evolution of annelids from pre-annelidan coelomate stock (Gardiner, 1978, 1979). A comparative structural and ultrastructural analysis of the tentacles and body wall of *Owenia* identified several primitive structures shared with lophophorates, echinoderms, and hemichordates ("archicoelomates" of European literature; Gardiner, 1978, 1979; Gardiner and Rieger, 1980).

An ultrastructural investigation of the organization of the larval nephridium of *O. fusiformis* was undertaken to compare it with the pore canal-hydropore complex (nephridium) of deuterostome larvae as described by Ruppert and Balser (1986). We present evidence that the nephridium of the mitraria is morphologically identical to the larval deuterostome nephridium and discuss both its function and relationship to the nephridia of deuterostome and non-deuterostome larvae.

## MATERIALS AND METHODS

Mitraria larvae of *Owenia fusiformis* delle Chiaje were obtained from plankton tows off of Pawley's Island, South Carolina. Mitraria larvae were identified as belonging to *Owenia* rather than to *Myriochele,* the other genus of the Oweniidae whose larvae are found in the plankton off of this coastline, by the number of cilia per cell. Cells of the mitraria of *Owenia* are monociliated, whereas cells of the mitraria of *Myriochele* are milticiliated. Specimens were relaxed in magnesium chloride isotonic to seawater and fixed for 48 h at room temperature in 2.5% glutaraldehyde in 0.2 *M* Millonig's phosphate buffer (pH 7.6) and 0.34 *M* sodium chloride. They were post-fixed for 1 h at room temperature in 1% osmium tetroxide in 0.34 *M* sodium chloride and 0.2 *M* Millonig's phosphate buffer (pH 7.6). Following post-fixation, specimens were dehydrated in ethanol, transferred through two changes of propylene oxide, and embedded in Polybed 812 (Polysciences, Inc., Warrington, Pennsylvania). One micrometer and thin sections were cut with diamond knives on a LKB Ultrotome Nova. One micrometer sections were stained with methylene blue. Thin sections were collected on bare copper hexagonal mesh grids, stained with alcoholic uranyl acetate and lead citrate, and examined with a Philips EM 300 transmission electron microscope. The terminology used for larval structures and their spatial orientation is based upon Wilson (1932).

## RESULTS

The mitraria larva of *Owenia fusiformis* possesses a single pair of nephridia. Each nephridium is situated in the blastocoel, lateral to the setal sacs (Figs. 1, 2A). Both nephridia attach to the larval episphere by the dorsal levator muscles and lie directly against the ventral epidermis of the hyposphere (Figs. 1, 2A). Each nephridium is approximately 40 $\mu$m in length and it ranges in height from approximately 12 $\mu$m proximally, at the point of attachment of the dorsal levator muscles, to approximately 6.5 $\mu$m distally, where it opens into the nephridioduct. The tubular nephridioduct extends anteriorly from each nephridium and opens to the exterior through the first segment of the developing worm rudiment. It averages 30 $\mu$m in length and 3.0 $\mu$m in diameter.

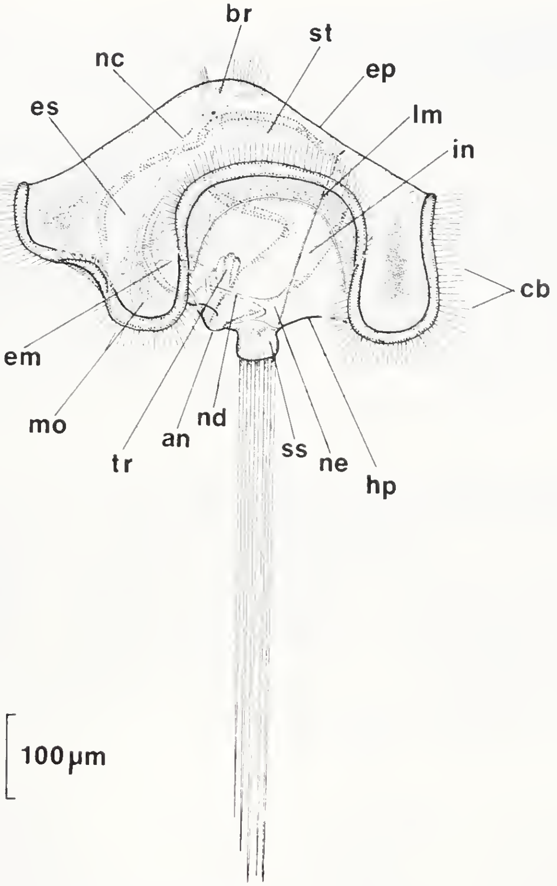

FIGURE 1. Diagram of the mitraria larva of *Owenia fusiformis* (after Wilson, 1932). an, anus; br, cerebral ganglion; cb, prototrochal ciliary band; em, esophageal muscle; ep, episphere; es, esophagus; hp, hyposphere; in, intestine; lm, dorsal levator muscles; mo, mouth; nc, circumesophageal nerve cord; nd, nephridioduct; ne, nephridium; ss, setal sac; st, stomach; tr, trunk rudiment.

Transmission electron microscopic observations indicate that each nephridial sac is lined by monociliated podocytes which lie on a basal extracellular matrix (ECM), averaging 90 nm in thickness (Figs. 2B–D; 3A). At the point of origin of the dorsal levator muscles from the nephridium, the nephridial lumen is lined by both podocytes and myocytes which are continuous with the levator muscle (Fig. 2B). Therefore, the levator muscle is a modified part of the nephridial lining as is the levator muscle in the tornaria larvae of hemichordates (Morgan, 1894; Ruppert and Balser, 1986). Each podocyte consists of a cuboidal perikaryon, 1.7–3.5 $\mu$m high, which bulges into the nephridial cavity (Fig. 2B–D), and attenuated, lateral foot processes (pedicels). Filtration slits between adjacent pedicels are 40–70 nm in diameter and lack diaphragms (Fig. 2D). Adhaerens junctions join the lateral surfaces of adjacent podocytes (Fig. 2E inset). The luminal surface of each perikaryon bears a single cilium and filiform microvilli. The cilium, which is oriented towards the duct, arises from a

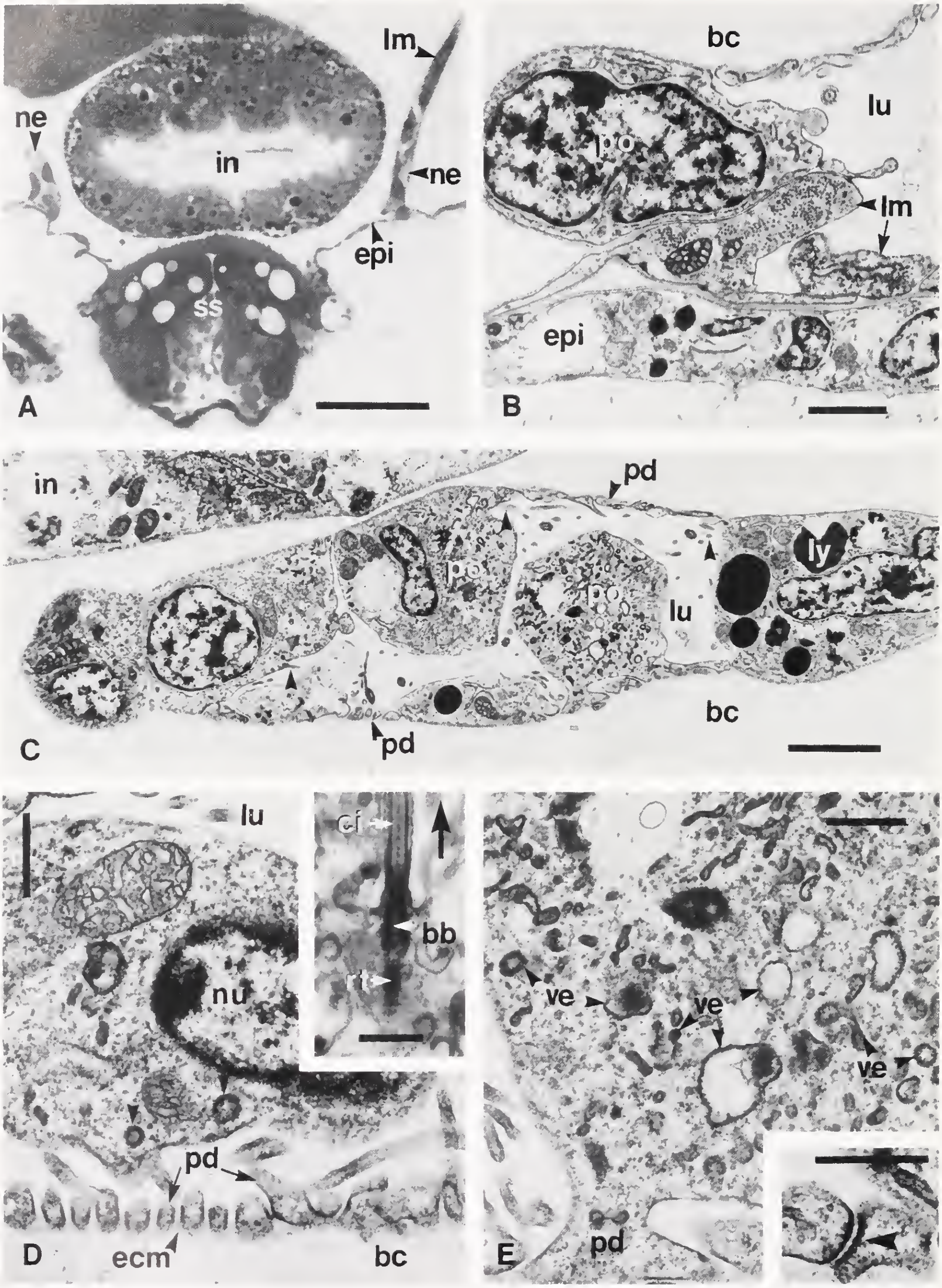
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FIGURE 2. Nephridium of mitraria larva of *Owenia fusiformis*. A. Light micrograph of an oblique frontal section through a mitraria larva showing a pair of nephridia (ne) situated within blastocoel of hyposphere. Each nephridium rests on the epidermis (epi) of hyposphere. Note origin of dorsal levator muscle (lm) from nephridium situated on the right. Scale = 20 $\mu$m. B. Transmission electron micrograph (TEM) of a longitudinal section through proximal most region of nephridium showing relationship of nephridial podocytes (po) to dorsal levator muscle (lm). The nephridial lumen is lined by both podocytes

shallow pit and has an accessory centriole and caudal rootlet at its base (Fig. 2D inset). The rootlet is oriented towards the duct (Fig. 2D inset). Smooth and coated endocytotic pits occur along the apical and lateral plasmalemmas of each cell body. Perinuclear endocytotic vesicles, endosomes, lysosome-like bodies, small Golgi complexes, mitochondria, and a few RER cisternae occur within the cytoplasm of each cell (Figs. 2C–E, 3A).

The nephridioduct consists of squamous monociliated cells, approximately 3.5 μm in height at the perikarya and 1.9 μm at their lateral margins. The duct cells rest on a basal ECM, 16–30 nm in thickness, (Fig. 3B) and adjacent cells are joined by lateral adhaerens junctions. Each duct cell wraps around itself and encloses an extracellular lumen, which is approximately 0.8 μm in diameter (Fig. 3B–D). An adhaerens junction joins the apposed surfaces of each doughnut-shaped cell (Fig. 3B). Each perikaryon bulges slightly into the lumen and bears a single subapical cilium and long filiform microvilli (Fig. 3B). The cilium emerges from a shallow pit, and an accessory centriole and caudal rootlet occur at its base (Fig. 3C). The ciliary rootlet, like the cilium, is oriented towards the nephridiopore (Fig. 3C). A few scattered smooth and coated endocytotic pits occur along the apical plasmalemma of the duct cells. Perinuclear endocytotic vesicles, endosomes, lysosome-like bodies, mitochondria, small Golgi complexes, and RER cisternae occur in each cell (Fig. 3B–D).

## Discussion

Polychaete larvae possess protonephridia with either monociliated (Goodrich, 1945; Holborow, 1971; Smith and Ruppert, in press) or multiciliated (Goodrich, 1945; Pemerl, 1965; Wessing and Polenz, 1973; Heimler, 1981; Smith and Ruppert, in press) terminal cells. The results of this study reveal that the nephridium of the mitraria larva of *O. fusiformis* is not a typical protonephridium, rather it is a small body cavity lined by monociliated podocytes which opens to the exterior by a ciliated duct. Although this organization is unique to *Owenia* among the annelids, it is identical to that of the hydropore, pore canal, and adjoining coelom of deuterostome larvae which also is a nephridium (Ruppert and Balser, 1986).

Two dominant types of nephridia are recognized in aquatic invertebrates, protonephridia and metanephridia. Although typically defined solely on their morphology and germ layer origin (see Goodrich, 1945; Wilson and Webster, 1974), they can also be defined functionally. A protonephridium is an excretory organ where cilia-mediated filtration occurs on the nephridial wall and a metanephridium is an excretory organ where muscle-mediated filtration occurs on blood vessels or their analogs

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and myocytes of the levator muscle in this region. Scale = 1 μm. C. TEM of a parasagittal section through nephridial sac. Note podocytes (po) lining nephridial lumen (lu) and pedicles (pd) and microvilli (arrowheads) extending from podocytes. Scale = 2 μm. D. TEM of a nephridial podocyte showing its pedicels (pd) resting on the underlying basal extracellular matrix (ecm) which forms the filtration barrier. Note perinuclear endocytotic vesicles (arrowheads) within its cytoplasm. Scale = 0.5 μm. Inset: TEM of single cilium (ci) of a podocyte and its associated basal body (bb) and rootlet (rt). An accessory centriole, although present, is not shown in this section. Arrow indicates direction of nephridiopore. Scale = 0.5 μm. E. TEM of a nephridial podocyte showing numerous endocytotic vesicles (ve) situated within its cytoplasm. Scale = 0.5 μm. Inset: Adhaerens junction (arrowhead) between two adjacent podocytes. Scale = 0.5 μm. bb, basal body; bc, blastocoel; ci, cilium; ecm, basal extracellular matrix; epi, epidermis of hyposphere; in, intestine; lm, dorsal levator muscle; lu, nephridial lumen; ly, lysosome-like bodies; ne, nephridium; nu, nucleus; pd, pedicel; po, podocyte; ss, setal sac; ve, endocytotic vesicles.

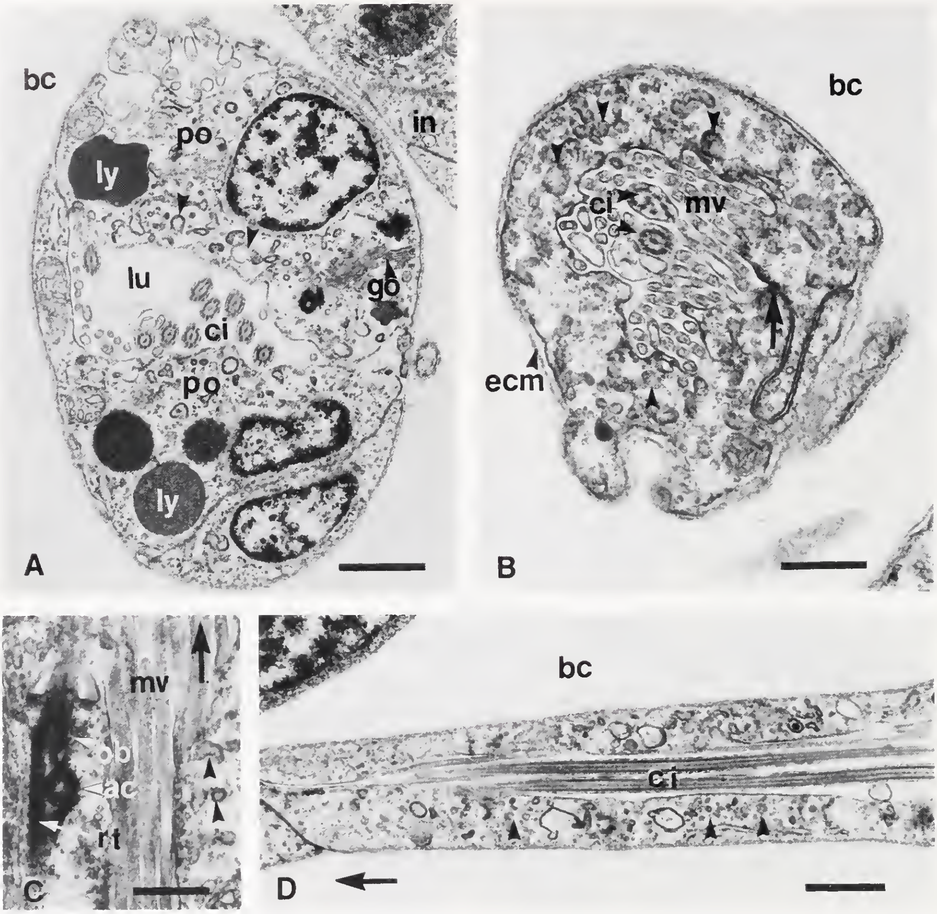

FIGURE 3. TEMs of nephridium and nephridioduct of mitraria larva of *Owenia fusiformis*. A. Transverse section through distal most portion of nephridium near point of union with nephridioduct. Note lysosome-like bodies (ly) within cytoplasm of podocytes. Scale = 1 μm. B. Transverse section through medial portion of nephridioduct. Note adhaerens junction (arrow) between apposed surfaces of doughnut-shaped cell and numerous endocytotic vesicles (arrowheads) within its cytoplasm. Cilia (ci) situated within its lumen extend from duct cells. Scale = 0.5 μm. C. Ciliary basal body (bb), rootlet (rt), and accessory centriole (ac) of a nephridioduct cell. Note microvilli (mv) within duct lumen and endocytotic vesicles (arrowheads) within cytoplasm of duct cell. Arrow indicates direction of nephridiopore. Scale = 0.5 μm. D. Parasagittal section through distal most portion of nephridioduct. Note endocytotic vesicles (arrowheads) within duct cell cytoplasm. Arrow indicates direction of nephridiopore. Scale = 1 μm. ac, acessory centriole; bb, basal body; bc, blastocoel; ci, cilia; ecm, basal extracellular matrix; in, intestine; lu, lumen of nephridioduct; ly, lysosome-like body; mv, microvilli; po, podocyte; rt, rootlet.

(Ruppert and Smith, 1985; in prep.). Based on these definitions, each mitraria nephridium is a giant protonephridium. Presumably the ECM between the podocyte pedicels forms the filtration surface while the podocyte and duct cilia produce the filtration pressure gradient. Blastocoelic fluid may be ultrafiltered across the ECM between the pedicels. Numerous endocytotic vesicles in the podocyte perikarya suggest that modification of the ultrafiltrate occurs within the nephridium. Additional modification may occur along the nephridioduct before the fluid is discharged at the nephridiopore. As suggested by Ruppert and Balser (1986) for nephridia of deuterostome larvae, osmotic fluid recovery across the body wall of the mitraria may be established by proteins in the blastocoelic fluid.

The pore canal-hydropore complexes contribute during metamorphosis to the metanephridial systems of adult hemichordates and possibly asteroid echinoderms (Ruppert and Balser, 1986), whereas the nephridium of the mitraria undergoes histolysis (Wilson, 1932) and is superseded by a pair of metanephridia in the sixth adult segment (setiger five).

Ultrastructurally, the mitraria nephridium is identical to those of larval enteropneusts and asteroids, but are they homologs or analogs? Consideration must be given to comparative larval anatomy and functional constraints on nephridial design in larval body organization to provide a tentative answer to this question. We have previously discussed the correlation of cilia-driven filtration nephridia (protonephridia) with the absence of blood vessels and the physical basis for the lack of circulatory systems in small animals, such as many larvae (Ruppert and Smith, 1985; in prep.). On the basis of functional considerations, protonephridia are expected in small animals.

Comparison of the mitraria nephridium with that of a tornaria, with which it seems directly comparable, reveals histological similarities and anatomical dissimilarities. The nephridia of both larvae are lined by an epithelium composed of monociliated podocytes and myocytes. The myocytes form an apical muscle band that traverses the blastocoel and inserts on the episphere, but the point of insertion is different in the two larvae. In the mitraria, insertion is posterior to the cerebral ganglion, whereas in the tornaria it is on the ganglion. Moreover, the mitraria nephridia are situated postorally and the nephridioducts open to the exterior through the first pair of segmental somites, whereas the nephridium and its dorsal duct in the tornaria are situtated preorally. In addition, the mitraria nephridia are paired while those of the tornaria are unpaired, although paired ducts, apparently atavisms, occur frequently in laboratory reared larvae (Gemmill, 1914; Ruppert, unpub. obs.).

We conclude that the striking cytological and histological similarities between the mitraria nephridia and those of asteroid and enteropneust larvae reflect a cellular homology, the common possession of monociliated podocytes. Like myocytes or neurons which also occur widely in metazoans, podocytes *per se* are of limited usefulness in the recognition of relationships. On the other hand, the anatomical differences cited above suggest mitraria nephridia, as organs, may not be homologous to those of hemichordates and echinoderms; at best they might be serial homologs. However, this does not weaken the archicoelomate affinities of *Owenia* because the mitraria nephridia compare favorably to the monociliated larval protonephridia of phoronids. Although Wilson (1932) rejected the correspondence of mitraria nephridia with typical protonephridia (head kidneys) of other polychaete trochophores, we suggest that the two are homologous and that the differences encountered between them may be explained in terms of larval body size (Ruppert and Smith, in prep.).

*Owenia* is viewed as being primitive within the extant Polychaeta because it possesses several characters which are believed to be plesiomorphic within the Metazoa. These include an unspecialized monociliated epidermis (Gardiner, 1978, 1979), a basiepidermal nervous system (Gardiner, 1978, 1979), and muscle cells with a single rudimentary cilium (Gardiner and Rieger, 1980). Do the larval nephridia further corroborate the view that *Owenia* is primitive among the polychaetes?

Polychaetes are, in basic structural design, vermiform, soft-bodied, segmented coelomates. Based on recent discussions of function, this implies that adult polychaetes rely on a hydrostatic skeleton for locomotion (Clark, 1964), a blood vascular system for a through-flow internal transport system (Ruppert and Carle, 1983), and a metanephridial system for excretion (Ruppert and Smith, in prep.). If an unsegmented small-bodied larva, such as a trochophore, is included in the basic polychaete life cycle, then on functional grounds it should have a protonephridial system (Ruppert and Smith, in prep.). Therefore, it is possible that the stem species of the Polychaeta had a larval protonephridium and an adult metanephridium. In that case, neither the protonephridium nor the metanephridium is primitive to the other, rather they are co-primitive organs in polychaetes, each of which may be modified in particular lineages (Smith and Ruppert, in press).

*O. fusiformis* possesses the nephridial design postulated above for the adult and larva of the polychaete stem species. The segmented adult of *O. fusiformis* possesses a blood vascular system and metanephridia and the mitraria larva possesses a protonephridial system. Both the metanephridia of the adult and the protonephridia of the mitraria are composed of monociliated cells. Rieger (1976) has shown that the presence of an epithelium composed of monociliated cells is a plesiomorphic feature within the Metazoa. This suggests that *O. fusiformis* has preserved the original nephridial designs of the adult (a monociliated metanephridial duct) and the larva (a protonephridium composed of monociliated podocytes) of the polychaete stem species, thereby corroborating *Owenia's* position as the most primitive of the extant polychaetes.

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# FEEDING ACTIVITIES OF TWO SPECIES OF *CLYPEASTER* (ECHINOIDES, CLYPEASTEROIDA): FURTHER EVIDENCE OF CLYPEASTEROID RESOURCE PARTITIONING

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## ABSTRACT

*Clypeaster rosaceus* and *C. subdepressus* have typical clypeasteroid feeding mechanisms and collect particulate material primarily by means of oral surface accessory podia with terminal suckers. They lack the specialized food collecting podia seen in sand dollars, and the poorly differentiated food grooves carry only part of the food material. Maps of surface ciliary currents are provided and it is shown that they are not part of the feeding mechanism. Feeding mechanisms are discussed with reference to clypeasteroid phylogenetics. *Clypeaster rosaceus* occurs in association with seagrasses (*Thalassia testudinum*) and feeds extensively on dead leaves. *Clypeaster subdepressus* occurs on coarse biogenic sands and selects the upper size fractions (66% greater than 400 μm) for ingestion. Both are essentially epibenthic in habit and lack specialized spines to keep sand and debris from the body surface. Instead, they secrete an extensive sheet of mucus which prevents particles from falling between the spines. The suckered accessory podia of *C. rosaceus* have a mean tip diameter of about 180 μm, those of *C. subdepressus* about 130 μm. Although *Encope michelini* may occur in mixed flocks with *C. subdepressus* or *Leodia sexiesperforata,* they all feed on different fractions of the sediment. Probabilities of podial-particle encounters are insufficient to account for these differences and it is suggested that the basis of resource partitioning between sympatric species is due primarily to differences in size of food collecting podia.

## INTRODUCTION

The Clypeasteroida is a large, diverse group of epibenthic and shallow burrowing echinoids ranging in size from the tiny fibulariids (<15 mm) to the huge plate-like clypeasterids (>180 mm). They are abundant in many tropical seas and extend into cold temperate regions (Mortensen, 1948). In a recent series of papers the morphology and feeding behavior of these urchins has been investigated. At the outset, Mooi and Telford (1982) accepted the prevailing hypothesis that sand dollars such as *Echinarachnius parma* (Lamarck), ingested fine particles sieved through the aboral spine canopy. However, particles substantially larger than could be accommodated by the sieve mechanism (100 μm) were found in the gut, and this suggested that some other mechanisms might be involved. In a study of *Echinocyamus pusillus* (O. F. Müller) Telford *et al.* (1983) developed a method for watching feeding from underneath, using a horizontally mounted microscope with an inclined mirror. By this means, direct observations of podial and lantern tooth activities were made of clypeasteroids in their natural orientation. Using similar methods in a re-examination of *Echinarach-*

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*nius parma* intended to determine the possible contribution of the oral surface to feeding, Ellers and Telford (1984) concluded that most, and perhaps all, food gathering was accomplished by oral surface podia. This cast the first serious doubt on the sieve hypothesis. According to Telford *et al.* (1985), another major objection to the sieve mechanism is the physical impossibility of ciliary currents transporting material as proposed by Goodbody (1960), Seilacher (1979) and, indeed, as we ourselves thought at one time (Mooi and Telford, 1982). An entirely different mechanism has been proposed by Telford *et al.* (1985), based on the podial collection of particles which may subsequently be fractured by the lantern teeth. Although Kier (1974) had previously commented on the presence of "crushed" material in clypeasteroid guts, and Timko (1976) specifically described lantern activity in *Dendraster excentricus* (Eschscholtz), the lantern was thought to be inactive during feeding in *Mellita quinquiesperforata* (Leske) (Lane, 1977). More recently, Telford and Mooi (1986) have described the restriction of clypeasteroid species to specific substrate types and within limited particle size ranges. There is sufficient difference in the types and sizes of podia (Mooi, 1986a, b) to form the basis for the resource partitioning which appears to take place between sympatric species (Telford and Mooi, 1986).

The above studies have included the typical, little-specialized *Echinarachnius parma* (Echinarachniidae); three species, *Encope michelini* L. Agassiz, *Leodia sexiesperforata* (Leske), and *M. quinquiesperforata* (Mellitidae), specialized for hydrodynamically active environments (Telford, 1983); and one species, *Echinocyamus pusillus* of a family (Fibulariidae) specialized by miniaturization. The research has concentrated particularly on two areas: (a) Those structures and activities implicated in the supposed sieve hypothesis, notably spine morphology and spacing, ciliary currents and sites of mucus secretion; and (b) those involved in the proposed podial feeding mechanism, notably podial diversity, distribution, and dimensions. Our data have pointed to a widespread reliance on the podial mechanism.

In this paper we extend our observations to the family Clypeasteridae. This family is part of the sister group to all other clypeasteroid families (Smith, 1984). Therefore, members of the Clypeasteridae are very important because their feeding behavior might reflect the ancestral condition for the rest of the Clypeasteroida. In spite of their great abundance, size, diversity, and probable ecological significance, no previous studies of feeding in any species of *Clypeaster* have been reported. Our study deals with *C. rosaceus* (Linnaeus) and *C. subdepressus* (Gray), both abundant in the Gulf of Mexico, Caribbean, and adjacent waters. We show that their feeding mechanism is fundamentally the same as that of other clypeasteroids, and relies on podial collection and the crushing action of the lantern. Further evidence from species distributions and podial data are offered in support of our hypothesis of resource partitioning. Finally, an account of ciliary currents is given, which indicates their uniformity and ubiquity among the Clypeasteroida and confirms that, as in other species, they are not involved in food transport.

## MATERIALS AND METHODS

Specimens of *C. rosaceus* were collected at several sites in the Florida Keys (Pigeon Key, Bahia Honda, Long Key) in the summers of 1982 and 1984, at depths of 2–5 m. Patches of *Thalassia testudinum* were found at all collection sites. At some sites, the turtle grass was so dense that only small, isolated areas of sand were visible. *Clypeaster subdepressus* was collected 2–3 miles off Pigeon Key, at depths of 10–15 m. The substrate consisted of coarse carbonate sand with conspicuous coral and shell debris and without significant plant cover. For live observation, specimens were maintained in the laboratory in running seawater on 10–15 cm of sediment from the collection sites. Other specimens were fixed in the field by injection of 20% formalin in seawater and immediate immersion in 10% formalin in seawater. These specimens were used for examination of gut contents. For measurement of podial tip diameters, specimens were relaxed by slow addition of ethanol and fixed as described above. Forty-eight hours after fixing, specimens were transferred to 3% formalin in seawater for storage. The natural buffering capacity of seawater is sufficient to prevent etching of specimens in dilute formalin for periods of several weeks. Substrate samples were fixed by addition of concentrated formalin to give a final dilution by volume of 5% in seawater. Additional bathymetric and distributional data were obtained from the collection records of the Florida Department of Natural Resources (FDNR, Marine Laboratory, Tampa), from collections in the United States National Museum (USNM, Smithsonian Institution, Washington), and from those in the Museum of Comparative Zoology (MCZ, Harvard University, Cambridge, Massachusetts).

Representative samples of primary and miliary spines from the aboral surface, oral surface, ambitus, and infundibulum were drawn with the aid of a camera lucida. Podia were classified as accessory, food groove and large food groove types (Mooi, 1986a, b) and tip diameters of relaxed specimens were measured by eyepiece micrometer. Plastic thick sections (approximately 1 μm) of spines and podia were prepared using metachromatic staining methods (toluidine blue, cresyl violet) to determine the distribution of mucus secreting cells, as described by Mooi (1986a).

Surface ciliary currents were made visible by carmine particles or black ink and mapped as described by Telford *et al.* (1985). Observations of feeding activity on the oral surface were made using an inclined front-silvered mirror and stereomicroscope mounted horizontally on a swinging boom, as previously described (Telford *et al.*, 1983; 1985; Telford and Mooi, 1986). Analysis of particle size-frequency distributions of natural substrates and of material collected from the food grooves and infundibulum, was performed using a slight refinement of the method described by Telford and Mooi (1986). Small samples were strewn on a microscope slide and all particles within several fields of view were drawn using the camera lucida. The drawings were then spread on a "Summagraphics" M1103 digitizing tablet and their greatest orthogonal diameters were measured and recorded directly into a computer. A 1-mm stage micrometer was drawn with the camera lucida at the same enlargement for calibration. After determination of mean dimensions, the particles were sorted into size classes (<24.9, 25–49.9, 50–99.9, 100–199.9, 200–399.9, 400–799.9, >799.9 μm). Elongation (Leeder, 1982) was calculated as the ratio of lesser diameter to greater diameter. This particle size analysis cannot follow standard sedimentological procedures because the samples collected from individual animals are far too small for sieve analysis. Substrate and food particle size-frequency distributions were compared by Chi square. All statistical determinations followed the procedures of Sokal and Rohlf (1981).

## RESULTS

The spines of these *Clypeaster* species are differentiated into two principal types, primaries and smaller miliaries interspersed between them. Both types vary in size, depending on their location on the test (Fig. 1). Histological examination showed that primary and miliary spines possess mucus secreting cells along their shafts and at their tips. In *C. rosaceus* the primary spines are alike in shape in all regions of the body. They range continuously in size from the small aboral spines (1.7 mm, length/width ratio 7.7) to the large infundibular spines (5.2 mm, l/w 12.6). Those of *C. subdepressus* are mostly shorter and more slender. The aboral primaries differ from the others in having slightly inflated tips. They are about 1 mm in length, with a

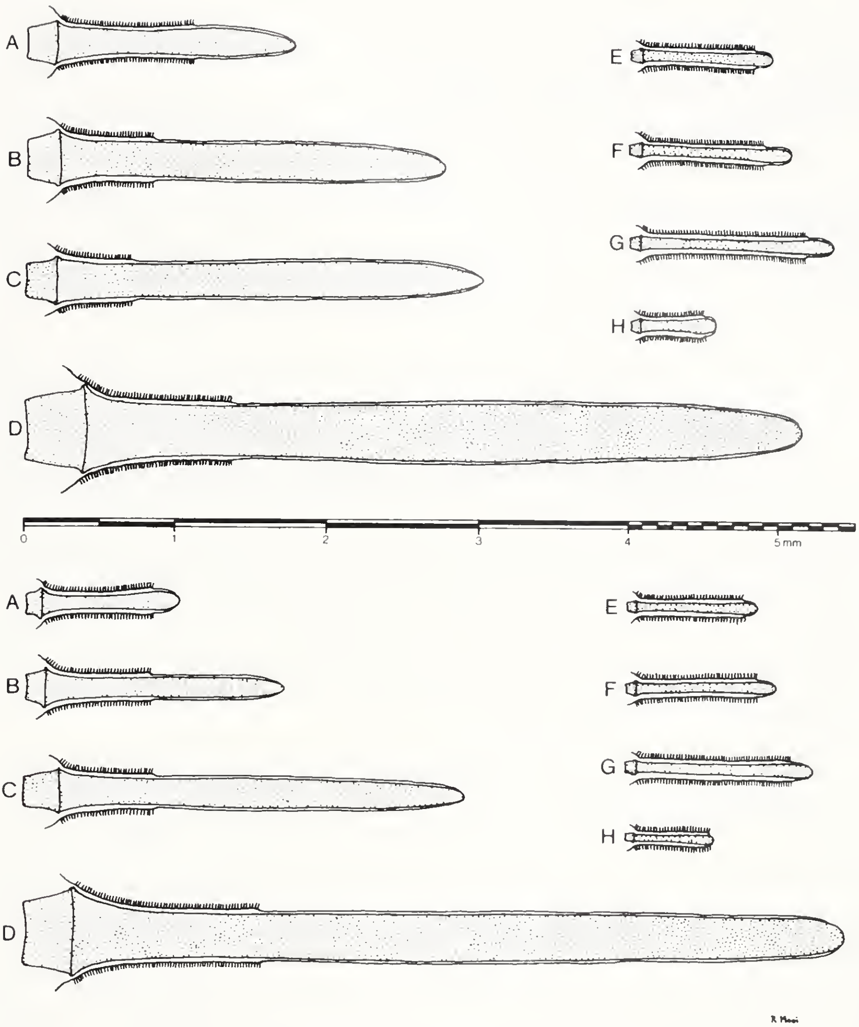

FIGURE 1. Spine types of *Clypeaster rosaceus* (above scale bar) and *C. subdepressus* (below scale bar). (A) aboral primary, (B) oral primary, (C) primary from around periproct (D) primary from inside infundibulum, near peristome, (E) aboral miliary, (F) oral miliary, (G) miliary from inside infundibulum, (H) miliary from food groove.

length/shaft-width ratio of 9.6 and a length/tip-width ratio of 6.6. In both species, the primary spines bear two well-defined rows of cilia which extend up to 1 mm from the base of the shaft. The miliary spines bear similar rows of cilia extending almost to the tip. The rows of cilia are easily discernible in living specimens as well as histological and SEM preparations. Unlike members of any other genus which we have examined, both species possess very small miliary spines, approximately 0.6 mm long, within the food grooves. Elsewhere, the miliaries range from 0.9 to 1.2 mm in length in *C. subdepressus* and are slightly longer in *C. rosaceus*.

Mooi (1986a, b) has described the histology and diversity of nonrespiratory podia in all clypeasteroid families. There are two principal types in *Clypeaster*, both of which bear terminal suckers. They are (1) accessory podia and (2) modified accessories or food groove podia. Examination of sectioned material showed these two types

TABLE I

*Mean diameters of podial tips (μm) for two species of* Clypeaster

| | *C. rosaceus* | | | *C. subdepressus* | | |
|---|---|---|---|---|---|---|
| | n | Mean | ±S.D. | n | Mean | ±S.D. |
| Aboral accessories | 90 | 199.9 | 10.35 | 90 | 145.0 | 7.86 |
| Oral accessories | 90 | 181.5 | 8.58 | 90 | 129.8 | 8.78 |
| Food groove (fg) | 60 | 166.1 | 10.27 | 60 | 140.3 | 9.42 |
| Large fg podia | 12 | 234.2 | 24.39 | 12 | 196.2 | 23.45 |

of podia to be well supplied with mucus secreting cells. Almost the entire oral surface supports accessory podia, with only narrow zones in the center of each interambulacrum lacking them. On the aboral surface they are distributed in triangular patches tapering from the ambitus into the petaloids. Accessory podia are highly extensible and very mobile. The food grooves are flanked by modified accessory (food groove) podia and have a narrow central area without podia along the suture line. Food groove podia are stubbier than the other accessories, but are none the less mobile and may extend to more than double their resting length. In adults the buccal podia are the first of some 10–15 pairs of considerably enlarged podia flanking each food groove adjacent to the mouth. These podia are extremely extensible and capable of reaching well beyond the tips of the infundibular spines. Table I provides measurements of tip diameters of accessory podia from three ambulacra as well as large food groove podia for one adult specimen of each species.

Ciliary currents in species of *Clypeaster*, although similar to those described for other clypeasteroids, are sufficiently different in some details to warrant reporting. Over the aboral surface the flow is essentially centrifugal (Fig. 2A). The petaloids are an exception where a counter current system exists between external ciliary flow and water-vascular fluid of the respiratory podia. On the oral surface of *C. subdepressus* (Fig. 2B) flow converges on the three anterior-most food grooves and follows them centripetally towards the mouth. Posterior to the peristome there is a very small zone of centripetal flow and an extensive area of flow convergent on the midline. This latter flow traverses the two posterior food grooves. Around the periproct (Fig. 2C) there is another area of convergent flow. In all areas of convergent flow there must, of course, be a change of direction away from the test (shown by circled dots in Fig. 2) to satisfy the principle of continuity (Vogel, 1981). As centripetal currents flow towards the center they, too, are constantly converging and must, therefore, have some flow exiting the spine field and directed away from the test. Within the infundibulum the centripetal flow reaches almost to the peristome and passes along the shafts of the circum-oral spines. As a result of this convergence, there is a downward flow towards the substrate, in the center of the infundibulum. The decreased velocity of flow in these areas of directional change can lead to a build-up of ink or carmine particles. In naturally oriented *Clypeaster*, the bulk of these particles fall away from the test onto the substrate below the infundibulum. Along the posterior interambulacrum and at the posterior margin of the periproct, convergent flows are mutually deflected towards the substrate, with similar loss of particulate matter suspended in these flows. On the oral surface of *C. rosaceus* (Fig. 2D), the centripetal flow in the broad infundibulum is more pronounced, including all five food grooves. The genus *Clypeaster* is the only one in which ciliary flow along the food grooves has been observed, and, as already remarked, it is the only one with miliary spines within the food grooves. Irregularities in the sediment surface and activity of the spines ensure

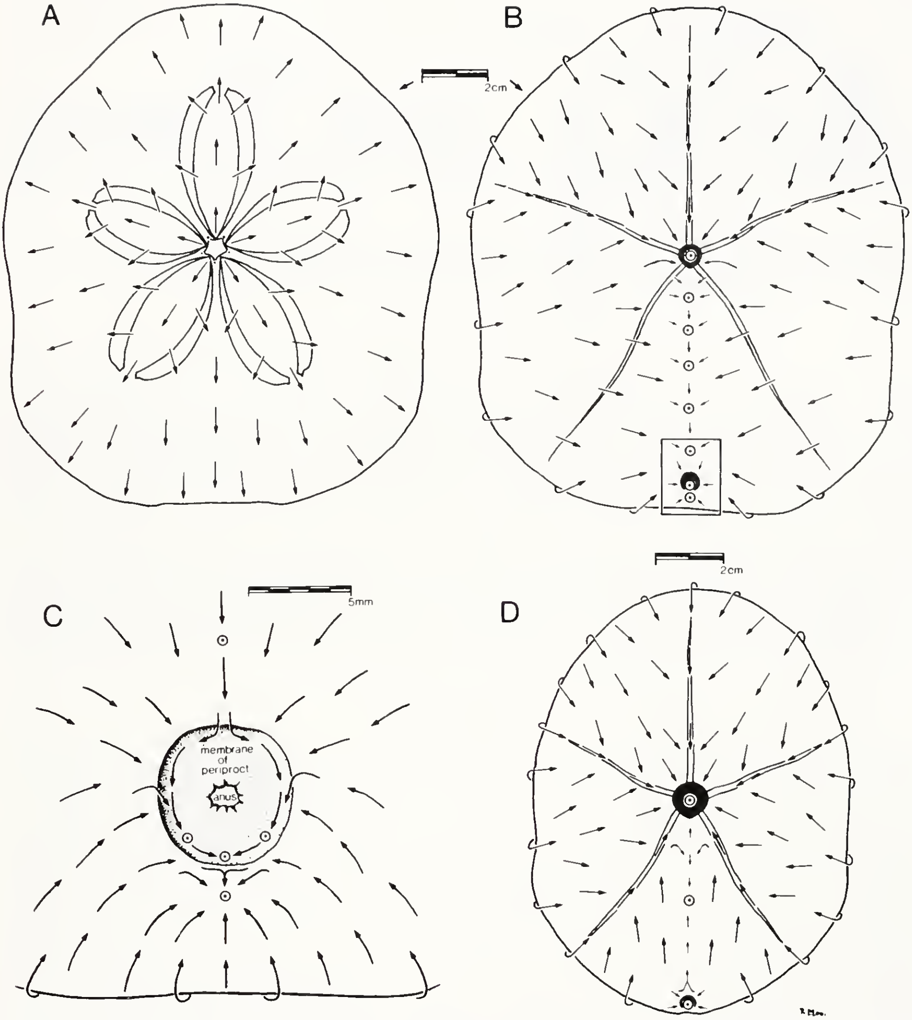

FIGURE 2. Ciliary currents on *Clypeaster subdepressus* (A–C) and *C. rosaceus* (D). (A) aboral surface, (B) oral surface, (C) area around periproct (boxed in B), (D) oral surface. In A, B, and D, large arrows indicate strong ciliary flow, smaller arrows weaker flow. In B to D, circles with central dot indicate downward flow towards the substrate in living specimens (towards the viewer in these diagrams).

that neither species is ever likely to be so closely in contact with the substrate as to seriously impede escape flow around the margin.

The two species considered here are very different in shape and behavior. When resting on a more or less flat surface (Fig. 3), *C. rosaceus* contacts the substrate around the edge and the broad, deep infundibulum places the mouth high above the surface. The rounded ambitus is not obscured by sediment particles but the aboral surface may be partially covered by dead filaments of *Thalassia* or other material (Fig. 3A), held in place by accessory podia. *Clypeaster subdepressus* is much flatter on the oral surface (Fig. 3B). Numerous particles picked up by podia around the ambitus are passed upward to the aboral surface by the combined action of spines and podia. On the aboral surface, particles move principally towards the posterior with a slight

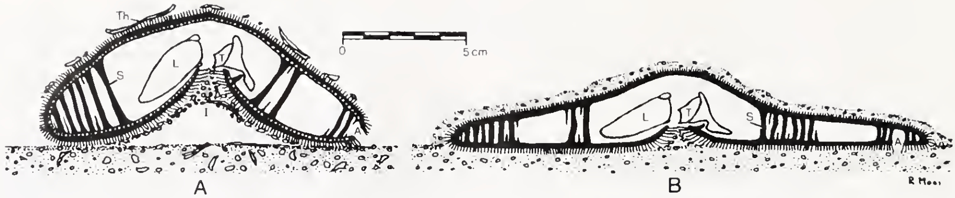

FIGURE 3. Diagramatic longitudinal sections through (A) *Clypeaster rosaceus* and (B) *C. subdepressus* showing epibenthic habit, degree of aboral surface cover, and relative positions of the peristomes. A, anus; I, infundibulum; L, lantern; S, test support pillar; T, tooth; Th, piece of dead *Thalassia.*

convergence on the midline. So far as we could tell, passage of material over the aboral surface was in no way related to the feeding activity of *C. subdepressus.* The aboral surface is largely covered by a thick mucus sheath secreted by the podia and spines. When the animals are resting or moving slowly, this sheath may be almost stationary but at times of greater activity it is moved steadily towards the rear by the action of spines, and it is replenished at the front and sides. Small sand grains and other fine particulate materials are trapped in the mucus, move with it, and are returned to the sediment as the sheet drops off the posterior edge. When complete, very few particles penetrate the sheet. Those which were pushed through with forceps, to fall among the spine bases, were rapidly moved to the ambitus by the combined action of pedicellariae and ciliary currents. The pedicellariae are so numerous that every spine is accessible to at least half a dozen of them.

During feeding, both species collected particulate material by means of oral surface accessory podia. Particles were then passed from podium to podium towards the mouth, sometimes without reference to the food grooves and sometimes along them. Transported particles moved up the slope of the infundibulum which, especially in *C. subdepressus,* became quite crowded with food material. The food grooves are rather unspecialized, the spines and podia flanking them being little differentiated from those adjacent to them. Small particles (100–200 $\mu$m) tended to become incorporated into indistinct mucus-bound strands; larger particles frequently moved alone but with adherent debris. On arrival at the peristome, particles were steered and propelled into the mouth by the large food groove podia and the circum-oral spines. Ingested particles were pulverized by the lantern teeth, which could be glimpsed periodically during observations of feeding. However, some particles, particularly large ones such as mollusc shells, were occasionally rejected. This was achieved by simply releasing the particle, or sometimes it was swiftly and methodically passed back to the ambitus against the prevailing centripetal particle movement.

Food material ingested by *C. rosaceus* included fragments of corals, coralline algae and large amounts of dead *Thalassia.* No attempt was made to determine the particle size-frequency distributions of substrate and ingestate of *C. rosaceus* because the presence of so much *Thalassia* vitiates the method. The material collected by *C. subdepressus* was analyzed and compared to the natural substrate (Fig. 4). The surrounding sediment was devoid of particles below 50 $\mu$m, had over 64% between 100 and 400 $\mu$m, and 35% larger than that. The mean grain size was 337.4 ± 200.9 $\mu$m with a mean elongation value of 0.69. This represents a poorly sorted sediment from which the very fine material has been removed by current activity. Within the food grooves and infundibulum, only 33% were in the 100–400 $\mu$m fraction and 26% were greater than 800 $\mu$m, compared to only 5% in the substrate. The mean grain size was much larger, 582.2 ± 417.6 $\mu$m. These differences are statistically highly significant ($P \ll 0.001$), but the elongation value (0.71) was not significantly different. The

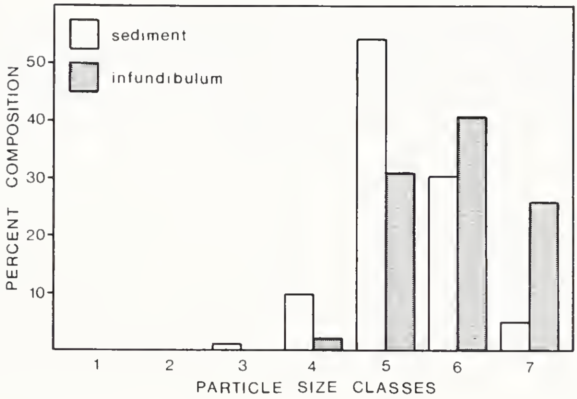

FIGURE 4. Percent composition of different size classes of particles in natural sediment at collection sites and of material collected from infundibulum of *Clypeaster subdepressus*. Size classes (1–7) were <25.0, 25.0–49.9, 50.0–99.9, 100.0–199.9, 200.0–399.9, 400.0–799.9, and >799.9 μm, respectively.

intestinal contents of *C. subdepressus* consisted mostly of a chalky paste with a few larger fragments. When the material is passed as feces, it is loose, not mucus bound and not particularly adherent. The stomach contents of *C. rosaceus* included pulverized material and conspicuous amounts of shredded leaves.

## DISCUSSION

*Clypeaster* species of the western Atlantic are found on biogenic sediments such as algal sands and shell debris (Serafy, 1979). According to Kier and Grant (1965), *C. subdepressus* occurs on substrates where it can burrow without interference from turtle grass roots. We never found this species properly burrowed. Usually individuals were extensively covered by substrate material but were always conspicuous, and never beneath the level of the surrounding substrate surface (Fig. 3B). The unspecialized spination of *Clypeaster* species and secretion of the mucus sheet both point to their essentially epibenthic habit. In *C. subdepressus* the aboral primary spines (Fig. 1A) are only slightly expanded at their tips, unlike the club spines of the truly burrowing mellitids (Telford *et al.,* 1985; Telford and Mooi, 1986). The miliary spines (Fig. 1E) have simple rounded tips and lack any crown-like expansions or terminal sacs (Mooi, 1986c). Both *C. rosaceus* and *C. subdepressus* produce copious amounts of mucus from spines and aboral podia. Mucus secretion by podia is a common occurrence among clypeasteroids (Mooi, 1986a, b). Goodbody (1960) suggested that the miliary spines of *Leodia* were sites of mucus secretion, but Mooi (1986c) has shown that only *Clypeaster* has mucus secreting cells in its miliary spines. The presence of mucus secretory cells in the primary spines further distinguishes the genus. In *C. subdepressus* the mucus covers much of the animal and forms a sheet or tent which traps fine and large particles. The mucus sheet prevents material from entering the spine field and is analogous in function to the canopy of club spines and miliary sacs found in the mellitids (Telford *et al.,* 1985; Telford and Mooi, 1986; Mooi, 1986c) and the mucus envelope of spatangoids (Smith, 1984). Beneath this protective covering, ciliary currents sweep away any stray particles that might chance to penetrate it. Observing an undisturbed animal from underneath, with its aboral mucus sheet in place, there are very few particles to be seen in the ciliary currents. The interspine spaces are kept scrupulously free of debris. Although extensive, the mucus cover on

*C. rosaceus* was not as complete as that of *C. subdepressus. Clypeaster rosaceus* was never found covered by sand, but it does cover itself with large pieces of shell and plant debris (Fig. 3A), as is the habit of many regular echinoids. Presumably its elevated shape and concave oral surface are adaptations to life within the beds of turtle grass. The wide, deep infundibulum provides space for the easy movement of long pieces of *Thalassia* and other food material, free of interference from living turtle grass and spines.

In the papers already cited, we have shown that the feeding mechanisms of several clypeasteroid species share features in common with other echinoid groups. Food material is collected and transported by the podia, in a manner analogous to that of spatangoids. In contrast to the spatangoids, both regular echinoids and clypeasteroids possess a fully functional Aristotle's lantern. The regular echinoids use the lantern to bite and scrape, whereas clypeasteroids use it to crush food material. The species of *Clypeaster* described in this paper are no exception. The great bulk of their food consists of particles collected and transported by oral surface accessory podia and it is thoroughly crushed by their large and powerful lanterns. We obtained no evidence that either species made use of fine material trapped in mucus. When carmine particles or black ink were being used to trace ciliary currents, it was apparent that some of the material reached the mouth area although it was often caught in mucus long before that. Some of it also became adherent to sand grains and was subsequently ingested. The mucus sheet covering most of the aboral surface traps fine material, all of which is returned to the substrate as the sheet trails off the posterior edge. As we have shown elsewhere (Telford *et al.,* 1985; Telford and Mooi, 1986) use of carmine and, to a lesser extent ink, can be misleading. A load of fine particles sufficient to be clearly visible vastly exceeds the amount normally encountered among the spines. It is possible that this stimulates mucus secretion. Furthermore, carmine particles are known to stick to almost any surface, including sand grains. We suspect that adhesion to the podia and some of the material in the food grooves is just such a passive effect, as we found with *Mellita* (Telford *et al.,* 1985), *Leodia* and *Encope* (Telford and Mooi, 1986). We cannot say categorically that ciliary borne material is not ingested, in fact some probably is. We can unequivocally state, however, that there is absolutely no evidence of its systematic collection; that remarkably little fine material is apparent in the sediment or in the ciliary currents and, finally, that the overwhelming bulk of the material ingested is collected by the podia. Field observations on these species by Kier and Grant (1965) are fully compatible with our findings.

Comparison of the size-frequency distributions of particles in the sediment and those from the infundibulum of *C. subdepressus* (Fig. 4) shows that large particles are collected with greater frequency than their occurrence in the sand. The oral surface accessory podia, responsible for collection of food, have suckered tips with a mean diameter of 130 $\mu$m (Table I). There appears to be a complex relationship between podia and particles which determines the composition of the material collected. Several factors are involved, including podial-particle encounters, handling efficiency, particle desirability, and the requirements for subsequent processing. The probability of a single podium encountering a grain of one particular size class can be estimated as the contribution of that size class to total sediment area. Thus, in a sample of natural sediment we would expect 54% of them to be in size class 5 (Fig. 4), for which the mean dimension is 300 $\mu$m. The product of grain proportion (0.54) and grain area ($300^2$) divided by the total area of all the grains is 0.2208. If particles were picked up in simple proportion to the frequency with which a podium encounters that grain size, then 22.1% should be in class 5, 40.8% in class 6 and 26.0% in class 7. Large particles are indeed favored, but simple encounter frequency alone cannot explain the observed size distribution of particles in the infundibulum. The relationship be-

TABLE II

*Percent by number of particles in natural sediment for habitat of* Clypeaster subdepressus *and estimated percentage by mass*

| Particle size | Observed % by number | Estimated % by mass |
|---|---|---|
| Class 3 | 1.0 | 0.0 |
| Class 4 | 10.0 | 0.2 |
| Class 5 | 54.0 | 9.1 |
| Class 6 | 31.0 | 44.6 |
| Class 7 | 4.0 | 46.0 |

Each size class represents twice the linear dimensions of the preceeding one. Mass is proportional to length$^3$, or eight times the mass of the next lower particle size class.

tween podial and grain dimensions is probably critical in particle handling. Thomas and Hermans (1985) have described the elegant duo-gland adhesive system responsible for attachment and release of the podia of a starfish. Mooi (1986a, b) has described the structure and action of the terminal suckers of clypeasteroid tube feet. Both processes, adhesion-release and suction, appear to operate in the collection and transport of particles. Our data suggest that particles of about five times the podium diameter might be optimal, and that below this size, effectiveness of the pick-up and transportation mechanisms diminishes. Larger particles could be handled cooperatively by several podia at once but above about twelve times the sucker diameter, effectiveness again falls off. No doubt particles are not equally attractive for reasons other than their size. Ellers and Telford (1984) observed that the presence of diatoms dramatically stimulated feeding in *E. parma,* and Telford *et al.* (1985) recorded a disproportionate accumulation of diatoms and foraminiferans in the food grooves of *M. quinquiesperforata.* It appears that sensory structures in the tube feet (Mooi, 1986a, b) enable clypeasteroids to discriminate between particles. Finally, the preferred size of particles collected might also be related to the dimensions and mechanics of the lantern apparatus where subsequent processing must take place.

It should be stressed that the data presented here consist of actual linear measurements of two dimensions and are therefore not directly comparable to sieve data. We have avoided using the standard sieve particle size classes, usually expressed in $\phi$ units, because that would invite spurious comparisons. It is apparent in Table II that when our data are transformed to approximate mass data (proportional to length$^3$), an entirely different impression of sediment composition is obtained. Grains are picked up individually during feeding and in our opinion frequency of particle size occurrence is more important in their selection than the accumulated mass passing through a screen, which is the form of data normally reported in sieve analyses.

Bathymetric range, geographical distribution, and the factors discussed above could provide the basis for resource partitioning by clypeasteroid species (Telford and Mooi, 1986). *Clypeaster subdepressus* has a bathymetric range of 5–210 m (Serafy, 1979) but most commonly occurs well below 50 m (Table III). Kier and Grant (1965) found it as shallow as 5 m, but mostly around 12 m, as we did ourselves. It inhabits coarse biogenic sand and we have been unable to find a reference to any *Clypeaster* species occurring on siliceous, terriginous sediments. This does not mean that some deep water species, such as *C. lamprus* H. L. Clark, could not occur on non-biogenic sediments. Although we found *C. subdepressus* co-occurring with *Encope michelini* at several localities, we never found it in association with *Leodia sexiesperforata.* However, the latter two species were often found in mixed flocks from 3–10 m on

TABLE III

*Bathymetric ranges, substrate preferences, and podial diameters of clypeasteroid species*

| Species | Common depth (m) | Natural substrate | | | Mean sizes ($\mu$m) | |
|---|---|---|---|---|---|---|
| | | Type | Sorted | Size ($\mu$m) | Food | Podia |
| *C. subdepressus* | 10–20 | c | p | 200–800 | 580 | 130 |
| *E. parma* | 0–50[1] | s | m | 100–400 | —[2] | 90 |
| *M. quinquiesperforata* | 0–4 | s | w | 100–400 | 170 | 70 |
| *E. michelini* | 3–10 | e | m | 100–400 | 180 | 100 |
| *L. sexiesperforata* | 2–5 | c | w | 100–400 | 140 | 70 |
| *E. pusillus* | 10–20 | s | p | >1000 | 350 | 60 |

[1] Stanley and James (1971).

[2] Method for collecting these data not developed at time of observation.

Unless otherwise indicated, original data can be found in reports of Telford and co-workers: See literature citations.

Substrate types were categorized as biogenic carbonates (c), terrigenic siliceous sediments (s), or either (e). Degree of sorting was ranked as poor (p), moderate (m) or well (w). The particle size range listed includes 70–90% of the total number of particles in the sample. Mean food size refers to material collected by the podia and transported towards the mouth directly or via the food grooves.

rather finer carbonate sands. As shown in Table III, *Leodia* generally occurs shallower than 5 m, *Encope* deeper. These bathymetric data from our field experience are very similar to those reported in the literature (Mortensen, 1948; Serafy, 1979; *inter alia*). For example, Kier and Grant (1965) found *Leodia* in 3–8 m depths, and they specifically commented on the preference of *C. subdepressus* for greater depths. It is quite apparent from these data and other collection records (USNM, MCZ, and FDNR) that *L. sexiesperforata* and *C. subdepressus* have almost completely separate bathymetric and substrate size ranges. *Encope michelini* occupies an intermediate range. It overlaps with *L. sexiesperforata* when that species is approaching its preferred depth limit and on relatively coarse sand, as we found at Torch and Long Keys, Florida. When *E. michelini* is itself near its preferred habitat limits, it overlaps with *C. subdepressus,* as at Pigeon Key, Florida. Detailed field data for all of these species are scarce, especially substrate analyses, and further evidence regarding their spatial separation is urgently needed. None the less, the data presented here and in Telford and Mooi (1986) indicate that within their broad geographical ranges, habitat preferences tend to keep the species separate (by bathymetric range, substrate type, and size, *etc.*). Where species do co-occur (*L. sexiesperforata* with *E. michelini* and *E. michelini* with *C. subdepressus*), differences in podial sizes affecting particle collection and transport, as well as lantern sizes affecting ingestion and crushing, cause them to feed preferentially on different particle fractions. This is of considerable ecological and biogeographical interest and has previously received very little attention. We have summarized all of the available data on common depth of occurrence, as well as particle and podial dimensions, in Table III. Generally, the mean food particle size is around two to five times the podial diameter and drawn from the most abundant particle size classes in the surrounding substrate. The exception is the minuscule *Echinocyamus pusillus* which has the smallest podia but lives in the coarsest substrate from which it selects relatively scarce small particles during feeding. This species does not actually swallow many of its food particles, it scrapes them with the lantern teeth and then releases them (Telford *et al.*, 1983). Unlike other clypeasteroid species, *Echinocyamus pusillus* lives in frequently disturbed environments, where it nestles between pebbles.

Populations of clypeasteroids are often dense and must play a significant role in reworking the sediment and recycling nutrients. The absence of very fine particles in many of the substrates examined suggests that clypeasteroid fecal material, released on or close to the surface, must be swept away by water currents or more rapidly dissolved as a result of increased surface area/volume ratios. *Clypeaster rosaceus* is specialized for existence among *Thalassia* plants and for a diet mostly of dead leaves. It is sometimes found adjacent to turtle grass patches in open sandy areas with *Encope,* where its diet is augmented by relatively large fragments of *Halimeda* and other coralline algal debris, pieces of coral and even bits of mollusc shell. Thus *C. rosaceus* also appears to feed mostly on resources not specially sought after by scutelline species with small podia. According to Kier and Grant (1965) it may also co-occur with *C. subdepressus.* Whether or not the two species then select essentially the same particle fractions is not known.

This study of feeding in *Clypeaster* species is also important because of its phylogenetic implications. The Clypeasteridae is part of the sister group (Clypeasterina) to the remaining members of the order Clypeasteroida (Smith, 1984). The feeding mechanism in *Clypeaster* is simpler and less refined than that of scutelline sand dollars such as *E. parma* (Ellers and Telford, 1984) or the mellitids (Telford *et al.,* 1985; Telford and Mooi, 1986). In *Clypeaster* the food grooves are simple, straight furrows and carry only a fraction of the food. In addition, they are unique in having small miliary spines along their length. In *Echinarachnius* and, more particularly, the Mellitidae, they are precisely defined, extensively branched, and convey all of the food to the mouth. Associated with the food grooves is the greater specialization of the podia. In *Clypeaster* there is little differentiation of the podia flanking the food grooves and those in adjacent areas. In the scutellines specialized podia actually occupy the food grooves and are solely responsible for moving food towards the mouth. Oral surface accessory podia in *Clypeaster* are not morphologically distinguishable from those of the ambitus or the aboral surface. In scutellines the oral surface bears barrel-tipped podia specialized for collection and transport of food material (Mooi, 1986a, b; Telford *et al.,* 1985; Telford and Mooi, 1986). In the most highly derived scutellines (the mellitids) these barrel-tipped podia are further divided into those specialized for gathering particles from the sediment and those for transporting them to the food grooves. Feeding by podial collection of sand also occurs in cassiduloids such as *Cassidulus caribbearum* (Lamarck) (Gladfelter, 1974) and *Apatopygus recens* (Milne-Edwards) (Higgins, 1974), which do so without the aid of food grooves. Since the cassiduloids represent an outgroup for the clypeasteroids (Smith, 1984), this feeding mechanism is plesiomorphic for the Clypeasteridae and culminates in the apomorphic, highly specialized podia and food grooves of the Mellitidae. The absence of such specialized structures in *Echinocyamus* (Fibulariidae) (Telford *et al.,* 1983) is an apomorphy associated with miniaturization and is not indicative of the ancestral condition.

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# MEMBRANE TRANSPORT OF PHOSPHATE BY *HYMENOLEPIS DIMINUTA*

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## ABSTRACT

The absorption of phosphate ion by *Hymenolepis diminuta* involved diffusion at all substrate concentrations tested. Mediated transport predominated at lower substrate concentrations and it exhibited saturation kinetics with $V_{max}$ and $K_m$ values of 0.056 $\mu$mol ethanol-extractable inorganic phosphate/g ethanol extracted dry wt/2 min and 1.19 m*M*, respectively, obtained from the Lineweaver-Burk transformation. The $V_{max}$ and $K_m$ values determined by the Hanes-Woolf plot were 0.136 $\mu$mol ethanol-extractable inorganic phosphate/g ethanol extracted dry wt/2 min and 3.64 m*M*, respectively. Absorption was influenced by changes in pH, temperature, and sodium ion concentration. Stable inorganic phosphate inhibited transport competitively. Various organic phosphates and common metabolic poisons introduced had no effect on transport. However, transport was inhibited by molybdate. From these data, it can be deduced that a separate transport system exists for phosphorus in *H. diminuta*.

## INTRODUCTION

Intestinal parasites acquire inorganic substances largely, but not exclusively, from the intestinal contents of their hosts (von Brand, 1973). Read (1950) showed that *Hymenolepis diminuta* accumulated appreciable amounts of $^{32}P$ *in vivo* only when the labelled inorganic phosphate (Pi) was given orally to the host under conditions ensuring that it reached the worm before being absorbed by the host tissues. However, a slow phosphate accumulation also occurred when it was administered to the host (rats) intraperitoneally (Read, 1950). Inorganic phosphate absorbed by parasites is generally incorporated rapidly into various organic compounds (Lesuk and Anderson, 1940; Scheibel *et al.*, 1968; von Brand *et al.*, 1969). Von Brand (1973) stated that there was reason to assume that several inorganic substances, including Pi, entered cestodes by diffusion since Daugherty (1957) discovered that the $Q_{10}$ of $^{32}Pi$ uptake by *H. diminuta* was very low. However, the authors were unable to find such information in the original article cited (Daugherty, 1957). Parasites usually grow at a much faster rate than free-living organisms, indicating that a large amount of Pi would be incorporated into phospholipids during membrane formation. Moreover, parasitic worms, like *H. diminuta,* produce enormous amounts of eggs containing many phosphorylated compounds. Hence, it would be inefficient for the growth and development of the parasite if it was to obtain such an important ion through diffusion. Therefore, the present investigation was undertaken to further elucidate the nature

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of the transport mechanism involved in phosphate absorption in the rat tapeworm, *H. diminuta.*

## MATERIALS AND METHODS

*Hymenolepis diminuta* was obtained from Carolina Biological Supply Co. (Burlington, North Carolina) as cysticercoids in adult *Tenebrio* sp. Male rats weighing 100 to 125 g were infected with 30 cysticercoids. Before and after infection, the rats were provided with water and food *ad lib.* Worms were flushed from the excised gut 10 days postinfection with Krebs-Ringer saline (KRT) containing 120 m*M* NaCl, 4.8 m*M* KCl, 2.6 m*M* $CaCl_2$, 1.2 m*M* $MgSO_4$, and 25 m*M* tris (hydroxymethyl)-aminomethane-maleate buffered at pH 7.4 (Read *et al.,* 1963). For all experiments where the ionic product of calcium phosphate can easily exceed its solubility product, calcium was deleted from the KRT buffer used for preincubation and incubation.

### *Identification of the ethanol-extractable radio-phosphate*

Experiments examined the nature of the ethanol-extractable inorganic phosphate after a two-minute incubation period. Samples of 0.5 g worms were preincubated in KRT buffer, followed by two minutes incubation in 0.2 m*M* labelled phosphate media. Upon removal from the incubation medium, the sample was rinsed rapidly in three changes of KRT buffer, blotted dry, and homogenized in six volumes of cold ethanol (80%). It was then centrifuged and the precipitate removed. To the supernatant fluid, 0.05 ml of 25% barium acetate was added to every milligram of inorganic phosphate in solution and the pH brought to 8.3 with sodium hydroxide. The resulting mixture was chilled and the precipitated phosphates collected by centrifugation, washed once with 70% ethanol, dried, and made into thick pastes with water. Dowex 50X8-200 ($H^+$) was added until dissolution was complete. The supernatant fluid was separated from the resin, adjusted to pH 4.5 with 5 *N* sodium hydroxide, reduced to a small volume of 25 $\mu$l, and used for paper chromatography and high voltage paper electrophoresis. The solvent system for paper chromatography was methyl cellosolve, pyridine, glacial acetic acid, and water (8:4:4:1), while 0.2 *M* borate buffer at pH 9.5 was used for high voltage paper electrophoresis. Electrophoresis was performed at 20V per centimeter for 45 min. Radioactive spots were located with a Berthold Beta Camera LB 292 (Berthold, Germany) and further determined by liquid scintillation counting of 0.5 cm strips of the processed paper.

### *Incubation studies*

Standard incubation conditions were two minutes at 37°C in a water bath shaking at 100 oscillations per minute. All samples were incubated in radioactive monosodium phosphate adjusted within a range of 0.04 to 1.50 $\mu$Ci/$\mu$mol depending on the nature of the experiment. For the concentration study (0.1 to 3.0 m*M*), a constant amount of $^3$H-polyethylene glycol (PEG 4000, NEN) of 1.39 $\mu$Ci was also introduced into the incubation media adjusted to 0.3 $\mu$Ci/ml. For all the other experiments, radioactive PEG was deleted from the incubation medium. Upon removal from the incubation medium the worms were rinsed rapidly in three changes of KRT buffer, blotted free of excess moisture on filter paper, and extracted overnight in tubes containing two milliliters of 70% ethanol. During the subsequent 24-h extraction, the tubes were agitated several times and one milliliter aliquots of the ethanol extracts were mixed with five milliliters of Biofluor (NEN) and radioactivity assayed using a Packard Tri-Carb 300 liquid scintillation spectrometer. Dry weights of ethanol-

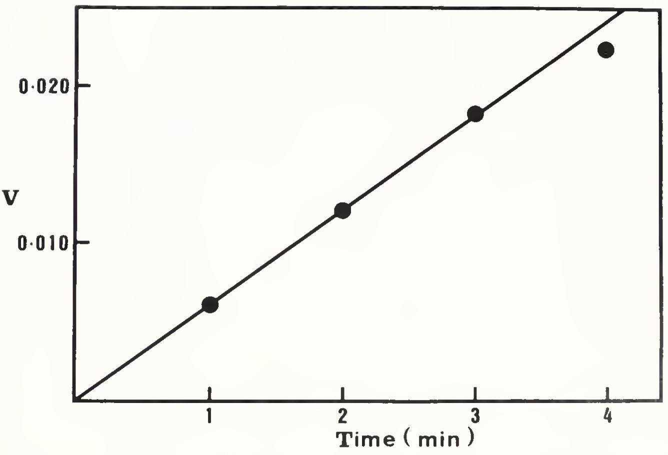

FIGURE 1. Absorption of 0.1 m*M* radiophosphate by *Hymenolepis diminuta* as a function of time (min). V = $\mu$mol/g ethanol-extracted dry wt/2 min. Each point is an average of three determinations. $r^2$ equals 0.87

extracted samples were obtained by heating worms at 110°C for five hours. Studies were also performed using balanced electrolyte solution (BES) containing: $Na^+$ 133 m*M*, $K^+$ 5 m*M*, $Mg^{++}$ 1.2 m*M*, $Cl^-$ 130 m*M*, $HCO_3^-$ 10 m*M*, mannitol 25 m*M*, and equilibrated with 5% carbon dioxide in nitrogen to pH 7.0 (Podesta *et al.*, 1977b); and balanced saline solution (BSS) containing: NaCl 120 m*M*, KCl 5 m*M*, $MgCl_2$, $NaHCO_3$ 1 m*M*, 275 to 280 mosmol/kg, and equilibrated with 5% carbon dioxide in nitrogen to pH 7.4 (Ip and Fisher, 1982). Calcium and phosphate were deleted from the original formulae.

### *Efficiency of ethanol extraction*

After ethanol extraction, worm samples were rinsed in 70% ethanol and digested in 0.4 ml of 60% perchloric acid and 0.8 ml of 30% hydrogen peroxide. The mixture was incubated at 70°C until colorless, cooled, and radioactivity assayed by liquid scintillation counting to determine the efficiency of extraction.

Monosodium of $^{32}P$-phosphate and $^{3}H$-polyethylene glycol were obtained from Amersham International plc (Amersham, United Kingdom). Reagent grade chemicals and stable phosphates were obtained from commercial sources.

Uptake velocities were expressed in micromoles of ethanol-extractable radioactive inorganic phosphate/g ethanol-extracted dry wt/2 min.

Student's *t* test was used to evaluate differences between means. Whenever applicable, linear regression was performed using the Apple II microcomputer.

## RESULTS

The absorption rate of 0.1 m*M* radiophosphate by *H. diminuta* expressed as micromoles of ethanol-extractable phosphate per gram ethanol extracted dry weight was constant over a period of four minutes (Fig. 1). Therefore, two minutes was chosen as a suitable incubation time for all incubation studies.

TABLE I

*Efficiency of ethanol extraction of absorbed radioactive phosphate*

| Conditions | Ethanol-extractable radiophosphate / Total radiophosphate absorbed |
|---|---|
| 0.1 m*M*, 2 min | 0.608 |
| 0.3 m*M*, 2 min | 0.542 |
| 0.5 m*M*, 2 min | 0.513 |
| 0.7 m*M*, 2 min | 0.533 |
| 1.0 m*M*, 2 min | 0.464 |
| 1.5 m*M*, 2 min | 0.548 |
| 2.0 m*M*, 2 min | 0.519 |
| 2.5 m*M*, 2 min | 0.561 |
| 3.0 m*M*, 2 min | 0.509 |
| 0.1 m*M*, 0.5 min | 0.521 |
| 0.1 m*M*, 1.0 min | 0.513 |
| 0.1 m*M*, 2.0 min | 0.489 |
| 0.1 m*M*, 10.0 min | 0.539 |

Each value is an average from two experiments.

### *Identification of the ethanol-extractable radio-phosphate*

Information on the identity of the ethanol-extractable phosphate was obtained by determining the ratio of radioactive inorganic phosphate to radioactive total phosphate in the ethanol extract. Results obtained by the various methods employed indicated that 95% of the extracted phosphate remained as the inorganic form.

### *Efficiency of ethanol extraction*

Efficiency of radioactive phosphate extraction by ethanol from *H. diminuta* is presented in Table I. It was found to be consistently about 50% of the total amount of radiophosphate absorbed disregarding the length of the incubation period and the concentration of the external phosphate.

### *Effect of different media on phosphorus absorption*

The absorptions of radiophosphate at various substrate concentrations tested in the BES and BSS were consistently lower than those in KRT (Table II).

TABLE II

*Effect of different media on radiophosphate absorption in* Hymenolepis diminuta

| Radiophosphate concentration (m*M*) | Rates of uptake in different buffers ($\mu$moles/g ethanol-extracted dry wt/2 min) | | |
|---|---|---|---|
| | KRT* | BES** | BSS*** |
| 0.1 | 0.0071 | 0.0033 | 0.0045 |
| 0.3 | 0.0201 | 0.0118 | 0.0107 |
| 0.5 | 0.0331 | 0.0127 | 0.0222 |
| 1.0 | 0.0547 | 0.0179 | 0.0308 |
| 2.0 | 0.0718 | 0.0217 | 0.0660 |

* Krebs-Ringer tris-maleate buffer (Read *et al.*, 1963).
** Balanced electrolyte solution (Podesta *et al.*, 1977b).
*** Balanced saline solution (Ip and Fisher, 1982).

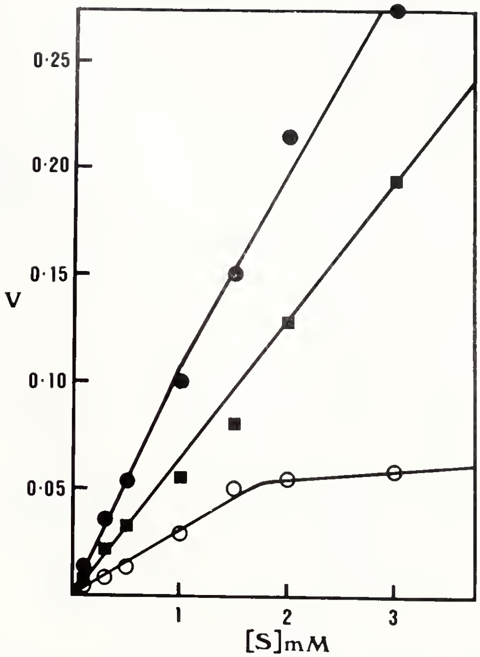

FIGURE 2. Absorption of radiophosphate by *Hymenolepis diminuta* as a function of phosphate concentration (0.01–3 m*M*). Each point is an average value from two experiments. S = phosphate concentration in m*M*; V = μmol/g ethanol-extracted dry wt/2 min. ● = Total uptake; ○ = mediated component; ■ = diffusive component.

### *Effect of substrate concentration*

The absorption process was a straight line relationship over a substrate concentration range of 0.03 m*M* to 20 m*M* suggesting diffusion might be involved. At lower substrate concentrations (0.01–3 m*M*) uptake was nonlinear and appeared to be a combination of mediated uptake and diffusion (Fig. 2). Subtracting the diffusive component obtained by deleting sodium from the incubation medium from the overall rate, the mediated process was unveiled with calculated $V_{max}$ and $K_m$ values of 0.056 μmol ethanol-extractable radioactive inorganic phosphate/g ethanol-extracted dry wt/2 min and 1.19 m*M*, respectively (Figs. 3, 4), obtained from the Lineweaver-Burk plot. Using Hanes-Woolf plot, the corresponding values of 0.136 μmol ethanol-extractable radioactive inorganic phosphate/g ethanol-extracted dry wt/2 min and 3.64 m*M* were obtained. From studies with $^3$H-PEG, it was found that after rinsing and blotting dry three times, only 0.5 to 0.6 μl of the incubation medium was carried over as unstirred layer per sample of worm (0.09–0.10 g ethanol-extracted dry weight). Since this was only a minute volume and it did not significantly affect the calculated results, the unstirred layer was not labelled in the subsequent incubation studies. With increasing concentrations of stable phosphate in the incubation medium, the initial rate of uptake of 0.5 m*M* labelled phosphate decreased and then levelled off (Fig. 5). This result supported the hypothesis that mediated transport was involved and further demonstrated a component of uptake that was not inhibited by increases in stable phosphate concentrations.

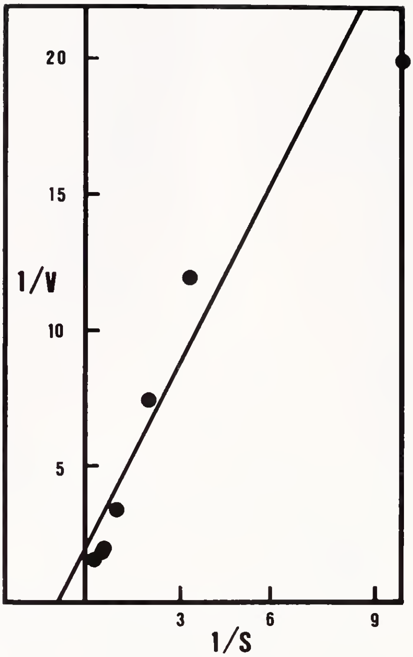

FIGURE 3. Lineweaver-Burk plot of radiophosphate absorption by *Hymenolepis diminuta*. V = $\mu$mol/g ethanol-extracted dry wt/2 min; S = phosphate concentration in m*M*. $r^2 = 0.96$.

*Effect of* $Na^+$

Worms were preincubated in various KRT media deficient in sodium, potassium, calcium, magnesium, chloride, nitrate, and sulfate ions. They were rinsed in three

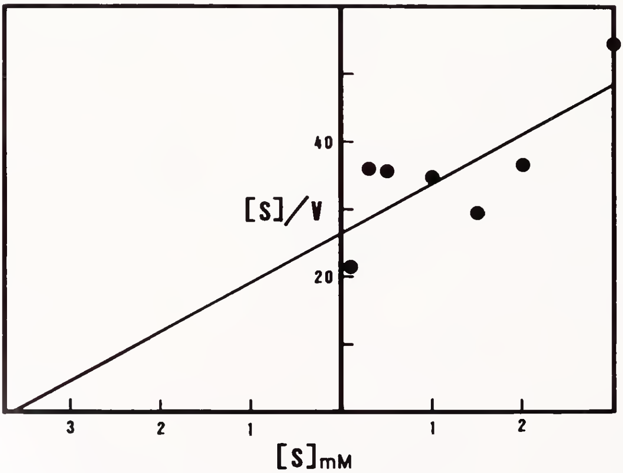

FIGURE 4. Hanes-Woolf plot of radiophosphate absorption by *Hymenolepis diminuta*. V = $\mu$mol/g ethanol-extracted dry wt/2 min; S = substrate concentration in m*M*; $r^2 = 0.61$.

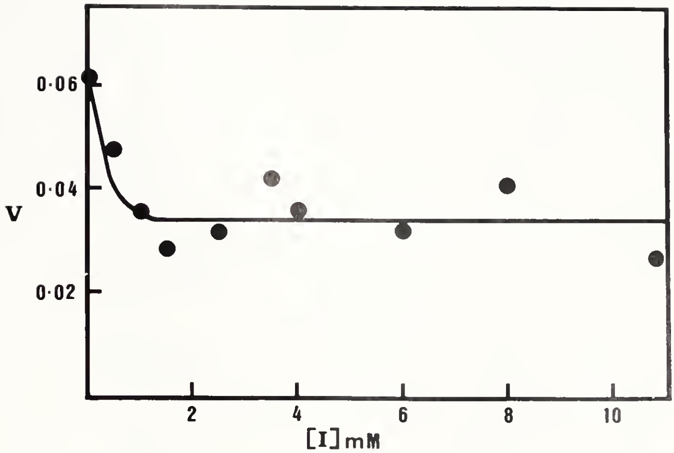

FIGURE 5. Effect of increasing stable phosphate concentration on uptake of 0.5 m*M* radiophosphate by *Hymenolepis diminuta*. Each point is an average value from two experiments. V = $\mu$mol/g ethanol-extracted dry wt/2 min; I = stable phosphate concentration in m*M*.

changes of the respective ion deficient buffer, blotted dry, and then incubated in 0.1 m*M* radiophosphate in the absence of the respective ions. Absorption of labelled phosphate was affected by $Na^+$ but not by the other ions tested. Therefore, worms were incubated in 0.1 m*M* phosphate with various sodium ion concentrations (using choline as a replacement for $Na^+$). Mediated phosphate uptake by *H. diminuta* was sodium-dependent and a hyperbolic function of $Na^+$ concentration of the ambient medium (Fig. 6).

### *Effect of glucose and methionine*

The absorption of 0.1 m*M* radiophosphate was not affected by the presence of either 1 m*M* glucose or 1 m*M* methionine in the incubation medium. The rates of absorption in the presence of glucose and methionine were 0.016 ± 0.002 ($n = 5$) and 0.018 ± 0.002 ($n = 5$) $\mu$mol ethanol-extractable Pi/g ethanol-extracted dry wt/2 min respectively, which were not significantly different from the control value of 0.019 ± 0.003 ($n = 5$, $P > 0.01$).

### *Effect of organic phosphates*

Fructose-6-phosphate (F6P), glucose-1-phosphate (G1P), guanosine triphosphate (GTP), uridine diphosphate (UDP), NADP, and ATP (concentration 10 m*M*) were used to determine the specificity of the transport system. Absorption of 0.1 m*M* radiophosphate was not affected by NADP and ATP but was inhibited by F6P, G1P, GTP, and UDP. However, when the worms were incubated in the latter organic phosphate esters together with ATP, no signs of inhibition were observed (Table III).

### *Effect of metabolic poisons and molybdate*

Sodium floride, potassium cyanide, parachloromercuribenzoic acid, iodoacetate, iodoacetamide, ouabain, dinitrophenol, and phlorizin were examined to determine

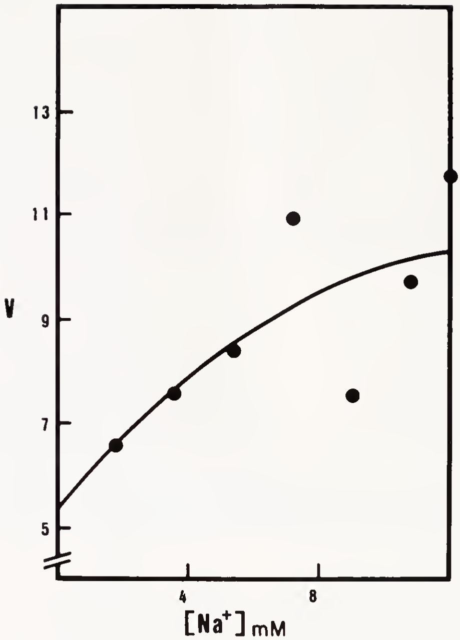

FIGURE 6. Effect of $Na^+$ concentration on mediated absorption of 0.1 m*M* radiophosphate by *Hymenolepis diminuta*. Each point is an average value from two experiments. V = $\mu$mol/g ethanol-extracted dry wt/2 min.

their effects on 0.1 m*M* labelled phosphate absorption. None of the above poisons had any effect on the uptake of phosphate by *H. diminuta* when they were added to both the preincubation and incubation media. Molybdate, however, inhibited the mediated uptake of radiophosphate by 72% when compared to the control value.

### *Effect of pH and temperature*

To study the effect of pH on the uptake of phosphate by *H. diminuta*, the worms were preincubated in KRT buffer at pH 7.4 and incubated for two minutes in 0.1

TABLE III

*Effect of various organic phosphates on the uptake of 0.1 m*M *radiophosphates by* Hymenolepis diminuta

| Inhibitor(s) (10 m*M*) | % Inhibition of mediated process |
|---|---|
| F6P | 30 |
| G1P | 28 |
| GTP | 23 |
| UDP | 41 |
| NADP | 0 |
| ATP | 0 |
| F6P + ATP | 0 |
| G1P + ATP | 0 |
| GTP + ATP | 0 |
| UDP + ATP | 0 |

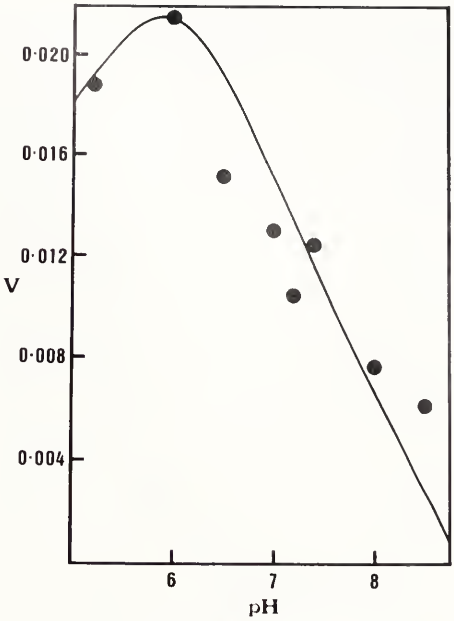

FIGURE 7. Effect of pH on the rate of 0.1 m*M* radiophosphate absorption by *H. diminuta*. Each point is an average value from two experiments. V = $\mu$mol/g ethanol-extracted dry wt/2 min.

m*M* radiophosphate at various pHs (5.2 to 8.5) (Fig. 7). The velocity of the absorptive process increased rapidly and peaked at pH 6. The effect of temperature on phosphate uptake was determined by incubating the worms in 0.1 m*M* radiophosphate in the presence or absence of external sodium at various temperatures. Incubation in $Na^+$ yielded results for both the diffusive and mediated processes (Fig. 8). However, incubation in $Na^+$ deficient KRT buffer abolished the mediated process, producing results only for the linear component. Hence, the difference between the two velocities of the uptake expressed results of temperature effect on the mediated process. Absorption of phosphate was optimal at 37°C (Fig. 9).

## DISCUSSION

The results of the short interval incubation experiments showed that 10-day-old *Hymenolepis diminuta* could absorb inorganic phosphate (Pi) from its surroundings. Ethanol could consistently extract only 50% of the radioactive phosphate absorbed (Table I) disregarding the incubation conditions. Even for an incubation period as short as 30 s, a similar large proportion of the absorbed phosphate was incorporated into the calcareous corpuscles and nucleic acids of the parasite (Ip, unpub. results). Since the time course study indicated that the free pool of inorganic phosphate within the worms, which was ethanol-extractable, did not attain a steady state relationship with the other internal phosphate pools and the external radioactive phosphate within four minutes of incubation indicating condition of initial uptake velocity (Table I, Fig. 1), the authors expressed the absorption rate in terms of micromoles of ethanol extractable radioactive phosphate per gram ethanol extracted dry weight per two minutes. More than 95% of the ethanol-extractable phosphate remained unmetabolized in the worm tissues.

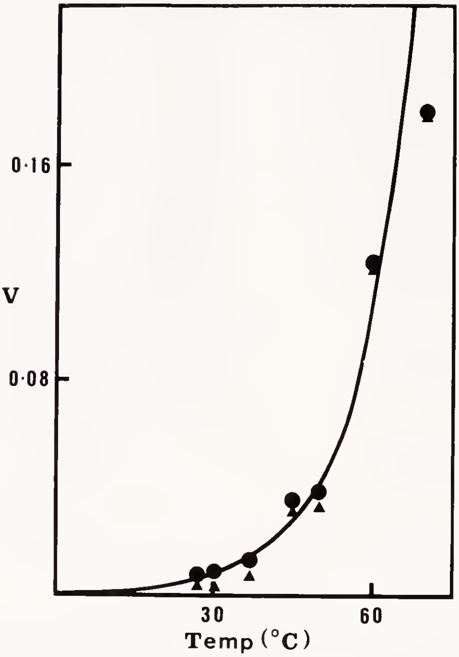

FIGURE 8. Effect of temperature on rate of total absorption and Na-independent uptake of 0.1 m*M* radiophosphate by *Hymenolepis diminuta*. Each point is an average value from two experiments. V = μmol/g ethanol-extracted dry wt/2 min. ● = Total uptake; ▲ = Na-independent uptake.

Krebs-Ringer tris-maleate (KRT) buffer was used in all incubation studies because of its ubiquity in other membrane transport studies of *H. diminuta*. It has been proposed that maleate in KRT may affect transport processes (Roth *et al.*, 1976). However, there are no clear indications that maleate specifically inhibits any trans-

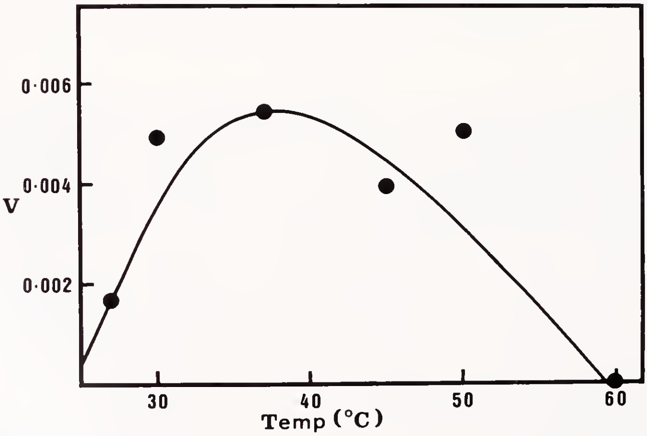

FIGURE 9. Effect of temperature on rate of mediated uptake of 0.1 m*M* of radiophosphate by *Hymenolepis diminuta*. V = mediated uptake of radiophosphate in μmol/g ethanol-extracted dry wt/2 min.

port process in *H. diminuta*, although Podesta *et al.* (1977b) reported an apparent reduction in the influx of glucose using KRT as the incubation media. Furthermore, the uptake rates of phosphate in KRT were considerably higher than those of balanced electrolyte solution (Podesta *et al.*, 1977b) and balanced saline solution (Ip and Fisher, 1982) (Table I). The adsorption of radioactive substance to the 'unstirred layer' of the brush border membrane of *H. diminuta* was minimum after rinsing and blot-drying, and did not impose serious errors on analyses of results for phosphate absorption. There was argument that the 'unstirred layer' would contribute errors to transport determination not only due to the adsorption phenomenon but also to the requirement of substance to diffuse across such barriers before becoming available to the transport mechanism (Podesta, 1977). Since the 'unstirred layer' is naturally present in the gut of the host *in vivo*, it was not the authors' intention to report $V_{max}$ and $K_m$ values of the mediated phosphate transport process under an artificial situation of no 'unstirred layer' effect, but to demonstrate a distinct phosphate transport mechanism different from those involved in other transport phenomena in *H. diminuta* under standard *in vitro* incubation conditions.

Phosphate uptake in *H. diminuta* consisted of two components: A saturable, $Na^+$ dependent component which obeys Michaelis-Menton kinetics and a $Na^+$ independent process which shows a linear relationship with increasing extracellular Pi concentration (Fig. 2). As suggested by Atkins and Gardner (1977), direct fit of the two term function $v = (V_{max}\{S\}/K_m + \{S\}) + k\{S\}$ (where v = micromoles ethanol-extractable radioactive Pi/g ethanol/extracted dry wt/2 min, $V_{max}$ = maximum velocity, $K_m$ = Michaelis-Menten constant, and k = the rate constant of the linear component) to the data obtained from concentration studies was performed. Subtraction of the rate of uptake of the $Na^+$ independent component (diffusion) from the overall rate yielded a corrected rate for the saturable process with $V_{max}$ and $K_m$ values of 0.056 $\mu$mol ethanol-extractable radioactive Pi/g ethanol-extracted dry wt/2 min and 1.19 m*M*, respectively, as obtained from the Lineweaver-Burk plot and corresponding values of 0.136 $\mu$mol ethanol-extractable radioactive Pi/g ethanol extracted dry wt/2 min and 3.64 m*M* as determined by the Hanes-Woolf plot. Such a discrepancy can be due to the inaccuracy of the Lineweaver-Burk determination of kinetic constants as there are relatively few points at the high end of the l/(S) scale, and it is these points that are most heavily weighted in such determinations. Moreover, small errors in the determination of $V_{max}$ are magnified when reciprocals are taken. The results of the present investigation contradict previous suggestion that absorption of Pi was due to diffusion (von Brand, 1973). Although diffusion occurred at all substrate concentrations tested, mediated transport predominated at substrate concentrations below 1.5 m*M* (Fig. 2). Mediated phosphate absorption was affected by the concentration of $Na^+$ in the ambient medium. This finding is consistent with that reported on the transport of inorganic phosphate transport by SV3T3 cultured cells (Brown and Lamb, 1975).

The cations and anions $K^+$, $Ca^{++}$, $Mg^{++}$, $Cl^-$, $SO_4^{--}$, and $NO_3^-$ had no effect on transport activity. The sodium requirement, as demonstrated in numerous other systems (Read *et al.*, 1974; Schultz *et al.*, 1974; Crane, 1977), suggests that sodium and phosphate may be cotransported by a catalytic protein 'carrier' system in *H. diminuta* similar to that proposed by Crane (1965) for glucose transport in mammalian intestine. Metabolic poisons, in particular ouabain, had no inhibitory effect on the rate of phosphate absorption in *H. diminuta*. Such a $Na^+$-coupled active transport system usually requires an ouabain-sensitive $Na^+$-$K^+$ activated ATPase (Schultz *et al.*, 1974; Crane, 1977). The observation that ouabain has no effect on glucose transport in *H. diminuta* (Lee *et al.*, 1963; Dike and Read, 1971b) raises doubt to the presence of such an enzyme in this parasite and its involvement in phosphate transport in the present study. However, *H. diminuta* is impermeable to ouabain (Gallogly, 1972) and the ouabain-sensitive $Na^+$-$K^+$ activated ATPase may not be localized in the surface membrane(s) of the brush border. Recently, the $Na^+$ extrusion mechanism was found to be localized below the tegument (Podesta *et al.,* 1977a) and this hypothesis is sustained by Lumsden and Murphy (1980). As such, the absence of any effect of ouabain on phosphate uptake in *H. diminuta* is expected. The mediated transport of phosphate was inhibited by molybdate, possibly the result of structural damage of the surface catalytic protein carrier molecules.

Arme and Read (1970) and Dike and Read (1971a) showed that phosphatase activity on the surface membrane of *H. diminuta* was totally inhibited by ATP. The absence of inhibitory effect when ATP was added to the incubation media in the presence of either F6P, G1P, GTP, or UDP indicated that these organic phosphates had no direct effect on inorganic phosphate absorption. The apparent inhibitory effect of these organic phosphates was a result of increasing inorganic phosphate concentration in the external media after hydrolysis of the respective phosphate esters by the surface enzyme. Since phosphate transport was not affected by a variety of organic phosphate esters, glucose, and methionine, it may be concluded that phosphate transport in *H. diminuta* occurs through a different mechanism with high substrate specificity.

The absorption of phosphate by the worm was sensitive to temperature and pH of the ambient medium. Optimal absorption occurred at 37°C. At lower temperatures, the surface enzymes required for mediated phosphate uptake are probably inactivated and at higher temperatures, the decreased rate is likely due to damage of the cestode integument. It is significant that maximum absorption occurred at pH 6 at which the major inorganic phosphate exists as $H_2PO_4^-$ in solution. Hence, diffusion can play a major part in phosphate uptake since this phosphate is less negatively charged as compared to the other possible phosphate ions.

## ACKNOWLEDGMENTS

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# INDUCTION OF PRECOCIOUS MOLTING AND CLAW TRANSFORMATION IN ALPHEID SHRIMPS BY EXOGENOUS 20-HYDROXYECDYSONE

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## ABSTRACT

Exogenous micromolar concentrations of 20-hydroxyecdysone were used to accelerate the molt cycle in the snapping shrimp, *Alpheus heterochelis.* Shrimps were exposed to the hormone by adding it to the seawater in which they were cultured. Five days of continuous exposure of an animal to a hormone concentration of 5 μgm/ml shortened the winter molt cycle length by 18 days, or 65%. Claw transformation, a commonly observed phenomenon in alpheid shrimps, also was accelerated at these concentrations, and postmolt animals exhibited appropriate modifications in external claw morphology and, in some cases, restructuring of the claw closer muscle according to schedules identical with those of animals having normal molt cycle lengths. These studies strengthen the hypothesis that the cellular events which underlie claw transformation are modulated by endogenous ecdysteroids.

## INTRODUCTION

The insect growth hormone, 20-hydroxyecdysone, promotes molting in a variety of crustaceans, whether directly injected into subject animals (Krishnakumaran and Schneiderman, 1969, 1970; Freeman and Bartell, 1976; Rao *et al.,* 1973; Tighe-Ford and Vaile, 1972) or added to the medium of cultured larvae (Cheung, 1974). Normally during the crustacean molting cycle, alpha ecdysone is thought to be synthesized and released from the Y-organ, and then converted to 20-hydroxyecdysone in target tissues (Skinner, 1985). The widespread and dramatic effects of these steroids has promoted the acceptance of ecdysones as generalized arthropod growth hormones.

We were interested in determining whether exogenous 20-hydroxyecdysone would be effective in shortening the molt cycle and in accelerating claw transformation in the snapping shrimp, *Alpheus heterochelis.* These intertidal crustaceans have extremely asymmetric claws—a large snapper and a small pincer—that take part in a remarkable morphological transformation: If the large snapper claw is caused to undergo autotomy, the pincer claw transforms into a snapper, while a new pincer regenerates at the original snapper site (Przibram, 1901; Wilson, 1903). Claw transformation, however, involves more than growth and change in external morphology. The two muscles in the propodite—the opener and the closer—enlarge considerably during transformation. Their motor neurons also become proportionally larger (Mellon *et al.,* 1981; Mellon and Smith, unpub. obs.) and their physiological and biochemical properties are modified (Stephens and Mellon, 1979; Quigley and Mellon, 1984). Moreover, a dramatic instance of cellular death accompanies transformation of the claw. In the pincer the closer muscle is a composite of two different fiber types: There is a central band of large fast type fibers that is flanked on either side by smaller

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diameter slower type fibers (Stephens and Mellon, 1979; O'Connor *et al.,* 1982). As the muscle transforms, the fast fibers die, while the slow fibers are modified to become very slow fibers (Quigley and Mellon, 1986). Muscle fiber death is apparent within a week following the first molt after onset of transformation; thus, both the change in claw morphology and the transformation of muscle fiber appear to be tied to the molt cycle, and both may be under hormonal control. To test this possibility, we exposed snapper-less shrimps to exogenous 20-hydroxyecdysone. The results show that the molt cycle can be reduced by as much as 65% in response to external ecdysone. Morphological claw transformation also was apparent in all of our experimental animals, while degeneration of the fast closer muscle fibers group occurred in two of the three experimental groups.

## MATERIALS AND METHODS

Experimental animals were recently molted, medium sized (4–5 cm) snapping shrimps (*Alpheus heterochelis*). Shrimps were housed individually in plastic containers in a 4 foot by 5 foot tray. Seawater entered the tray at one end, passed through the shrimp cages, and exited at the other end of the tray. Within a day of molting, animals were removed from the culture system and isolated individually in plastic containers with 100 ml artificial seawater (Forty Fathoms, Marine Enterprises Inc., Towson, Maryland).

The animals were exposed to exogenous hormone by adding to an individual animal's plastic container 100 microliters of a stock solution containing either 0.05 mg/ml or 0.5 mg/ml of 20-hydroxyecdysone (Sigma, E-2003). The stock solution was originally prepared by dissolving the hormone in 5% ethanol to the desired concentration.

Three different experimental protocols were used in our experiments. In the first protocol, shrimps were taken on the day following a molt, caused to autotomize the snapper claw, and placed immediately in the hormone bath. Hormone exposure was limited to five days, following which the animals were put into plain artificial seawater.

The second protocol involved removal of the snapper on the day following a molt and then waiting five days before exposure to hormone. In the third protocol, animals were left undisturbed for six days after molting at which time their snapper claws were removed. The animals were then placed in the hormone bath.

Regardless of the protocol, the animals were fed on the tropical fish food Tetramin every other day after which the bath water was changed. Molt cycles of animals exposed to the hormone were compared with those of animals kept in our culture system and from which the snapper claw had been removed two days following a molt. These control animals were obtained throughout the year, which was arbitrarily divided into winter (October–March) and summer (April–September). Our experiments were performed during February and March or during October and November. Data from the two groups were pooled. Room temperatures during the experimental periods varied between 22° and 24°C. Room lighting was diurnally supplemented through large glass windows. Seasonal variations in day length thus may have been sensed by the experimental animals.

The molt cycle of crustaceans has been divided into stages by various workers (Skinner, 1985). These differ qualitatively even across different groups of decapods. While there may be a correlation with the various molt cycle stages and the level of ecydsone in the hemolymph, this has not been measured in any shrimp species. In the crayfish *Orconectes,* however, both *in vivo* and *in vitro* measurements of ecdysone production suggest that the hemolymph titer begins to rise during the early stages of premolt and reaches a peak in late premolt (Jegla *et al.*, 1983). The blood hormone level then falls to low levels just prior to ecdysis.

We measured the molt cycle lengths in 36 snapper-less summer shrimps, 28 snapper-less winter shrimps, and in 43 ecdysone-treated snapper-less winter animals. Molt cycle length was taken as the number of days between two successive ecdyses.

Immunohistochemical procedures for examining biochemical and structural changes in the closer muscles of transforming claws have been described previously (Quigley and Mellon, 1986). Briefly, transforming claws were removed from animals eight days following the first molt after snapper claw autotomy. The claws were immediately frozen in melting isopentane and sectioned on a cryostat at 30 micrometers thickness. Frozen sections were mounted on glass coverslips and either stored at −80°C, or they were immediately reacted with monoclonal antibodies developed in our laboratory against fast and slow shrimp muscle myosin heavy chains. Muscle degeneration was assessed by microscopic examination of the stained, antibody-reacted sections. Fast fiber histolysis was presumed to have occurred when the fast-specific antibody reaction could no longer be detected in the central regions of the closer muscle. Usually, the absence of fast muscle fibers was also indicated by a tissue-free space in the central muscle. In any case, the reaction of adjacent frozen sections with antibody to slow fiber myosin assured that the tissues and reaction mixtures were normal.

## RESULTS

Figure 1 illustrates various stages in the morphological transformation of pincer to snapper. Not all stages are observed in any one animal during transformation. The earliest obvious morphological clue that transformation of the claw is occurring is the appearance (after a molt) of a mesial ridge on the dactylus. This ridge represents the earliest expression of the plunger on the fully transformed dactyl. We used the occurrence of any of the stages in Figure 1 as an indication that morphological transformation of the claw was occurring.

Our data, shown in Figure 2, compare molt cycle lengths in snapper-less summer and winter shrimps, and in ecdysone-treated snapper-less winter animals. On average, winter (October–March) molt cycles in transforming shrimps lasted about 29 days, while transforming summer (April–September) animals molted about every 23 days. This finding is similar to other studies of seasonal molt cycle change (*e.g.*, Freeman and Bartell, 1975). The underlying physiological reasons for the longer length of molt cycle in winter animals are not known; room temperatures in general were about two degrees higher in the summer, but other factors, such as day length, may play a significant role in determining growth and molting.

It is clear that treatment with 20-hydroxyecdysone at the concentrations we employed significantly shortened the usual winter molt cycle. Treatment in the experimental groups of animals was started either in February or early October, and experiments continued for two months. Normal, snapper-less animals from this time period would have had a molt cycle length of about 29 ± 3.8 days; those treated for five days with 20-hydroxyecdysone at the lower concentration we employed had a molt cycle of about 21 ± 4.8 days, while at the higher concentration the effect was more dramatic and the molt cycle length was only 10.5 ± 1.5 days.

Three different experimental protocols were used in exposing the experimental animals to the higher concentration of exogenous hormone. Figure 3 is a diagram illustrating these application schedules and the consequent effects upon a "normal" molt cycle length of 29 days. Shrimps undergoing protocol #1 molted, on average, 10.5 days after their previous molt, thus shortening their normal intermolt interval by 65%.

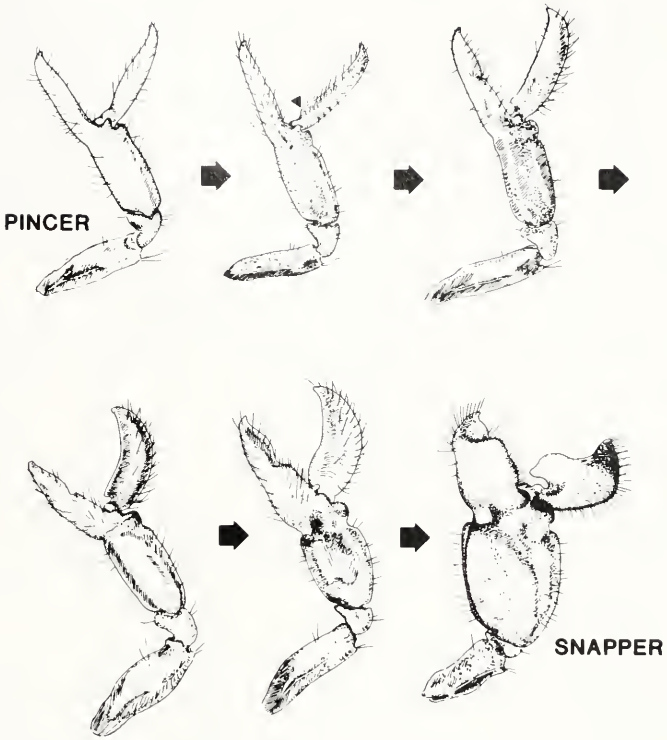

FIGURE 1. Morphological stages in the transformation of a pincer claw to a snapper claw. As a rule no single animal successively exhibits all of the stages illustrated, and in some instances, the stage exhibited at the first molt during transformation is considerably advanced. The caret indicates the earliest stage observed in the formation of the plunger found on the dactyl of the fully formed snapper.

Shrimps in the other two protocols molted roughly 14 days following their previous ecdysis, shortening their intermolt interval by 52%. Thus, the maximum reduction in molt cycle length was obtained by beginning hormone treatment immediately following the previous ecdysis.

It was of interest whether hormone-induced precocious molting and the accompanying claw transformation would elicit the modifications in claw closer muscle structure that normally occur in snapper-less shrimps. Therefore the claws of all surviving, transforming experimental animals were subjected to immunohistochemical analysis, as described previously (Quigley and Mellon, 1986).

Table I indicates the incidence of muscle degeneration in each of the three experimental groups. While two of the groups exhibited a preponderance of muscle remodeling, the group in which hormone treatment was initiated directly after a molt gave no evidence of closer muscle degeneration even though, in every case, the external morphology of the claw was clearly changing.

## DISCUSSION

A previous study (Freeman and Bartell, 1976) demonstrated that 20-hydroxyecdysone will shorten the molt cycle in another caridean shrimp, *Palaemonetes pugio.* Some differences are apparent between the results of that study and of our own exper-

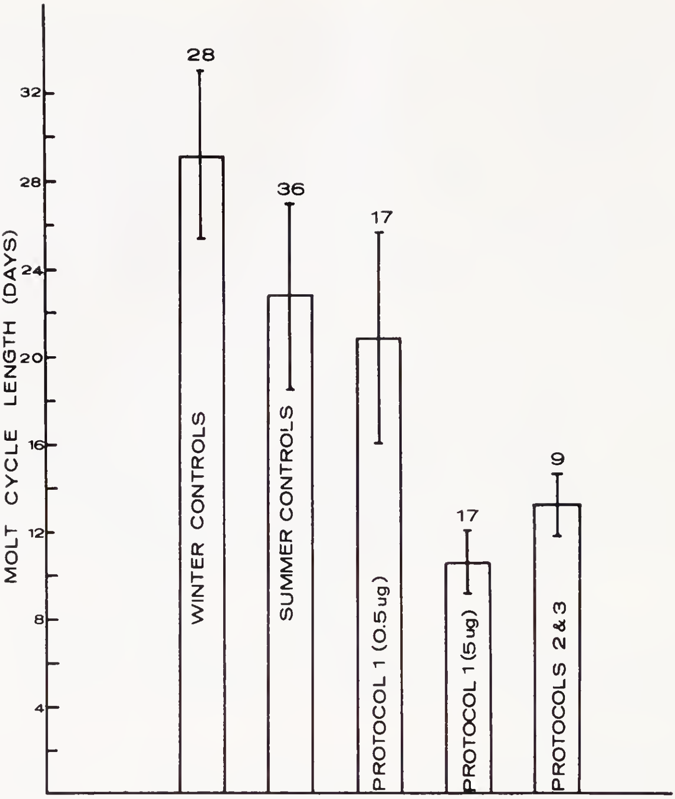

FIGURE 2. Bar graphs illustrating mean molt cycle duration in several groups of shrimps: Winter controls; summer controls; experimental animals in protocol 1 exposed to either 0.5 micrograms per milliliter or 5 micrograms per milliliter hormone concentration, and the pooled data from experimental animals in protocols 2 and 3, both of which were exposed to hormone concentrations of 5 micrograms per milliliter. Brackets at the top of each bar are ±one standard deviation, and numbers of animals in each group are shown above the brackets.

iments. In the study by Freeman and Bartell (1976), 20-hydroxyecdysone was injected into the experimental animals. Four different dosages were tried, and the lowest effective dosage (0.5 μgm/animal) shortened the intermolt cycle by 67%. Only 19% of this group survived the succeeding molt, however, whereas the overall survival rate in our three experimental groups was 72%. If the two shrimp species are physiologically comparable, it appears that simply bathing in the hormone is as effective a treatment as injection and is less traumatic to the experimental animals.

It is clear from our studies that exogenous 20-hydroxyecdysone accelerates not only the molt cycle in snapper-less *Alpheus*, but also the morphogenetic events that bring about transformation of the claw from a pincer to a snapper. This includes, in some cases, the programmed death of the fast group of muscle fibers in the claw closer. Fast fiber death was not seen in any of the experimental animals in protocol #1, in which hormone treatment was started one day following the preceding molt. The underlying physiological reasons for this failure are not known. In many decapod crustaceans the concentration of ecdysteroids in the hemolymph begins to rise in the premolt phase of the molting cycle, reaches a peak in mid premolt, and falls precipitously in late premolt, just prior to ecdysis (Jegla *et al.*, 1983; Skinner, 1985).

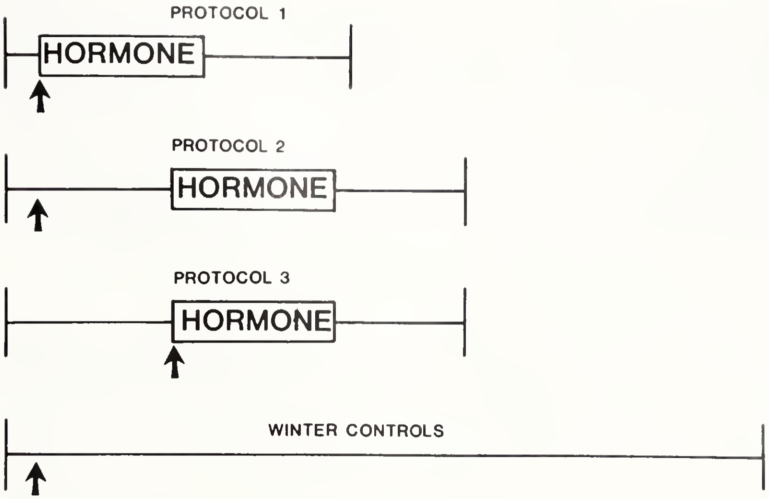

FIGURE 3. Relative molt cycle durations in winter control animals and in the three experimental protocols. Periods of hormone exposure are indicated, and arrows delineate the time point at which snapper claws were removed in each group.

If the hemolymph titers of ecdysone in *Alpheus* approximate this schedule, then it is apparent that the experimental animals in the first protocol may have been subjected to two distinct pulses of ecdysone, as opposed to the single, rather prolonged pulse which probably occurred in protocols #2 and #3. Since onset as well as reduction in blood hormone levels may act as a trophic signal (*e.g.,* Morton and Truman, 1985), the two pulses could have unpredictable consequences. Until the relative blood concentrations of ecdysone are precisely known throughout the intermolt period in *Alpheus,* however, further speculation cannot be justified. It is sufficient to conclude that, with an appropriate waiting period following molting, 5 days of treatment with exogenous ecdysone will shorten the mean normal molt cycle by more than 18 days, or by about 65%, and will proportionally accelerate the normal processes of claw transformation, including remodeling of the closer muscle. The procedure thus may prove to be useful as a tool in future experimental manipulations.

The mechanism of uptake (if any) of ecdysone from the bathing medium by the shrimps is not known. The gills of these intertidal shrimps are probably permeable to water and some inorganic ions, but it does not necessarily follow that molecules the size of steroids can pass into the blood through this route. Possibly, the hormone is ingested when the animals feed.

TABLE I

*Effects of experimental hormone treatments on closer muscle restructuring in transforming claws*

| | Total number of animals | Number of successful molts* | Number of closer muscles examined | Number of restructured muscles |
|---|---|---|---|---|
| Protocol 1 | 30 | 27 | 10 | 0 |
| Protocol 2 | 6 | 6 | 3 | 2 |
| Protocol 3 | 14 | 5 | 3 | 2 |

* Attrition was due either to death of the animal or to autotomized pincer claws.

Other possibilities must also be considered. We have observed that snapping shrimps held in communal culture systems, while physically separated from one another, tend to molt concurrently, suggesting the ecdysone or some other pheromone excreted into the culture water may be synchronizing their individual cycles. Such synchronization may be important to these gregarious, but cannibalistic crustaceans. In any event, the concentration of pheromone in the water must be very small. If this is the case, the shrimps may possess specialized external chemoreceptors that respond specifically to ecdysone, and through which molting could be triggered.

## ACKNOWLEDGMENTS

We are grateful to Dr. W. Otto Friesen for a gift of 20-hydroxyecdysone, and to W. D. Nelms Creekmur for technical assistance. The illustrations in Figure 1 were executed by Ms. Donna Bennett. This research was partially supported by an inhouse grant from the University of Virginia.

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# A TRYPSIN INHIBITOR FROM THE COELOMIC FLUID OF THE SEA STAR *ASTERIAS FORBESI*

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## ABSTRACT

Cell-free coelomic fluid obtained from the sea star *Asterias forbesi* rapidly neutralized, in a dose-dependent fashion, trypsin derived from several different animal species. Approximately 50 to 300 $\mu$g of bovine trypsin was inhibited per mg of total sea star coelomic fluid protein. Crude preparations of sea star and sea urchin trypsins isolated from gastric tissue, as well as purified human plasmin, were also neutralized upon addition to echinoderm coelomic fluid. In contrast, bovine $\alpha_1$-chymotrypsin and thrombin were not inactivated by the coelomic fluid inhibitor. The trypsin inhibitor was only mildly sensitive to heat or acid treatments and exhibited a molecular weight of ~6500. The protease inactivator was also detected in cell lysates derived from washed coelomocytes collected in N-ethyl maleimide, as well as in cell-free coelomic fluid collected by non-surgical means.

## INTRODUCTION

Trypsin represents a constitutive protease of most organisms, including bacteria (Winter and Neurath, 1970; Okabe and Noma, 1974; Russo and Yadoff, 1978; Goldberg *et al.*, 1980; Zwilling and Neurath, 1980). Trypsin inhibitors also have been detected within a wide variety of organisms, including invertebrates (Greene *et al.*, 1976; Tschesche, 1976; Tschesche and Dietl, 1976; Holzman and Russo, 1978). The invertebrate trypsin inhibitors inactivate sundry enzymes, including chymotrypsin. In the present communication, the detection and partial characterization of a sea star coelomic fluid inhibitor, which inactivates trypsin but not chymotrypsin, is described.

## MATERIALS AND METHODS

All chemicals and reagents were purchased from either Sigma Chemical Co. (St. Louis, Missouri) or Fisher Scientific (Pittsburgh, Pennsylvania) and were reagent quality or better. Synthetic substrates were obtained from Helena Laboratories (Beaumont, Texas). Bovine thrombin was purchased from Parke-Davis (Morris Plains, New Jersey). Sea stars (*Asterias forbesi*) weighing ~150 gm were obtained daily from the Supply Department of the Marine Biological Laboratory (Woods Hole, Massachusetts) and used only once.

Coelomic fluid was obtained by surgically amputating the tips of sea stars' arms and draining the fluid into chilled, pyrogen-free glass tubes. After centrifugation at $100 \times g$ for 5 min at 4°C, the supernate was decanted and represented cell-free coe-

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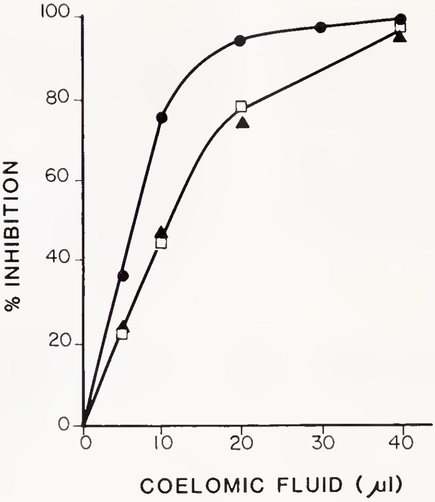

FIGURE 1. Inhibition of bovine trypsin by sea star cell-free coelomic fluid. Increasing amounts of coelomic fluid were pre-incubated with 0.1 μg of bovine trypsin for 10 min at 22°C (410 μl, final volume). Residual proteolytic (- ● -) activity was quantitated with the chromogenic substrate S2222 as described in Materials and Methods. Similar studies were conducted with coelomic fluid after heating to 100°C for 5 min (- □ -) and after acidification to pH 2.5 for 1 h at 24°C and neutralization (- ▲ -).

lomic fluid. The pellet, consisting of coelomocytes, was suspended in pyrogen-free, distilled water (one ml per animal). The cellular pellet was disrupted by frequent and vigorous shaking for 2 h at 22°C. Cellular debris was removed by centrifugation at 1000 × *g* for 10 min at 22°C. The supernate was decanted and represented the cell lysate. Phase contrast microscopy revealed that >90% of the cells were lysed under these conditions.

To measure the inhibitor activity of sea star cell-free coelomic fluid or cell lysate, residual enzymatic activity was quantitated employing chromogenic substrates. The assay was initiated by mixing coelomic fluid or cell lysate (5–400 μl) with 10 μl of enzyme for 10 min at 22°C (410 μl total volume). Controls were constructed by substituting the environmental buffer, 0.45 *M* NaCl in 50 m*M* Tris-HCl, pH 7.5, for the test sample. Chromogenic substrate (100 μl) was added to the above solution at a final concentration of 0.5 m*M*, and the reaction mixture was incubated for 5 min at 22°C and quenched with 200 μl of glacial acetic acid. Amidolysis was quantitated by reading the absorbance at 405 nm. The fraction of inactivated enzymatic activity was calculated by dividing residual amidolytic activity in either coelomic fluid or cell lysate by total enzymatic activity determined in the absence of these samples.

Protein concentrations were quantitated by the procedure of Bradford (1976) using bovine serum albumin as standard (Sigma, fraction V).

## RESULTS AND DISCUSSION

Addition of bovine trypsin (0.1 μg) to cell-free coelomic fluid resulted in rapid neutralization of the enzyme in a dose-dependent fashion (Fig. 1). As little as 5 μl of the sea star coelomic fluid inhibited ~40% of the added protease. The specific activity of the inhibitor under these conditions ranged from 50 to 300 μg of trypsin inhibited per mg of total coelomic fluid protein (8 animals were tested). As shown in Figure 1,

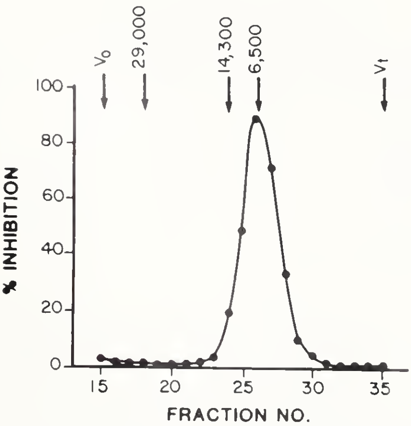

FIGURE 2. Gel permeation of sea star cell-free coelomic fluid. Coelomic fluid (200 μl) was gel filtered on Sephadex G-50 (0.6 cm × 115 cm) equilibrated in 0.45 *M* NaCl in 50 m*M* Tris-HCl, pH 7.5. Fractions of 0.9 ml were collected at a column flow rate of 9 ml/h. Inhibition of bovine trypsin by eluate fractions was determined by measuring residual enzymatic activity with S2222 as outlined above. Molecular weight standards included carbonic anhydrase (29,000), lyzozyme (14,300), and aprotinin (6500).

the inhibitor is only mildly sensitive to heat and acid treatments. A molecular weight of about 6500 was estimated by gel permeation chromatography using Sephadex G-50 (Fig. 2). The above molecular characteristics of the coelomic fluid inhibitor are similar to those described for other small molecular weight inhibitors (Fritz and Krejci, 1976; Tschesche, 1976; Tschesche and Dietl, 1976).

Crude preparations of proteolytic enzymes were obtained from the gastric tissues of *A. forbesi* and the sea urchin *Strongylocentrotus droebachiensis.* Briefly, the gut was excised surgically and disrupted with a glass pestle and homogenizer (Radnoti Glass Technology, Inc., Monrovia, California 91016). Cellular debris was removed by centrifugation at 1000 × *g* for 20 min at 4°C, and the supernate containing the gut enzyme(s) was decanted. These gut proteases represented trypsin-like enzymes based upon the following criteria: (1) Enzymatic activity was inhibited completely by bovine pancreatic trypsin inhibitor and soybean trypsin inhibitor, and (2) the proteases hydrolyzed synthetic peptide substrates containing carboxy-terminal arginyl residues (data not shown). Admixture of protease from either sea star or sea urchin with sea star coelomic fluid resulted in rapid inhibition of the respective enzymatic activity (Table I). Inhibition of these trypsin-like enzymes was dose-dependent, with ~40% of the enzymatic activity being inactivated by 5 μl of coelomic fluid (data not shown). Plasminogen was purified from human plasma (Deutsch and Mertz, 1970) and activated with streptokinase (Marcum *et al.,* 1982). Plasmin was rapidly neutralized upon addition to sea star coelomic fluid (Table I).

In contrast, bovine $\alpha_1$-chymotrypsin was not neutralized by the sea star coelomic fluid inhibitor (Table I). As little as 10 ng of the protease was not inactivated upon addition to sea star coelomic fluid (200 μl). The above data indicates that the specificity of the protease inhibitor is distinct from the Kunitz inhibitor which neutralizes trypsin and chymotrypsin (Kunitz and Northrop, 1936), but similar to the Kazal inhibitor which inactivates trypsin but not chymotrypsin (Kazal *et al.,* 1948). The majority of invertebrate inhibitors described to date are Kunitz-type inhibitors, whereas only the bdellins isolated from leeches are Kazal-type inhibitors (Fritz and

TABLE I

*Inactivation of enzymes by coelomic fluid inhibitor*

| Enzyme[1] | % Inhibition |
|---|---|
| Bovine trypsin | 100 |
| Sea star trypsin | 100 |
| Sea urchin trypsin | 100 |
| Human plasmin | 100 |
| Bovine $\alpha_1$-chymotrypsin | 0 |
| Bovine thrombin | 0 |

[1] Residual proteolytic activity was quantitated utilizing chromogenic substrate S2222 for measuring 0.1 μg bovine pancreatic trypsin (12,400 BAEE units/mg), S2238 for 0.1 unit bovine thrombin (56 NIH units/mg), S2586 for 0.01 to 1 μg bovine pancreatic chymotrypsin (50 BTEE units/mg), S2444 for 5.6 μg sea star gut-extracted protease(s) (197 BAEE units/mg), and S2238 for 3.3 μg sea urchin gut-extracted protease(s) (149 BAEE units/mg). In the case of human plasmin (1 μg), enzymatic activity was measured employing $^{125}$I-labeled α-casein as substrate (Highsmith and Rosenberg, 1977).

Krejci, 1976). It must be noted, however, that Kazal inhibitors inactivate thrombin, whereas the sea star inhibitor did not neutralize a crude preparation of bovine thrombin (Table I).

To determine whether the inhibitor is present in coelomocytes, coelomic fluid was collected in N-ethyl malemide (5 m*M*, final concentration), a potent stabilizer of plasma membranes (Bryan *et al.*, 1964). Analysis of washed coelomocytes collected under the above conditions revealed that lysate preparations of these cells also contained the trypsin inhibitor. However, the specific activity of lysates obtained from washed coelomocytes (5.89 μg trypsin inhibited/mg total protein) was ~20-fold less than the specific activity of the cell-free coelomic fluid (90 μg trypsin inhibited/mg total protein). These results suggest that the trypsin inhibitor is a natural component of coelomocytes and possibly synthesized by these cells and secreted into the coelomic fluid.

Experiments conducted with cell-free coelomic fluid, which was collected by mechanical manipulation without damage to sea stars, revealed that trypsin inhibitor levels within the above fluid were comparable to amounts quantitated for coelomic fluid obtained by surgical means. Bovine trypsin was inhibited in a dose-dependent fashion, with ~40% of the protease inactivated by 5 μl of the coelomic fluid (data not shown). The specific inhibitor activity of coelomic fluid collected by non-surgical methods (250 μg trypsin inhibited/mg total protein) was comparable to those obtained by surgical removal of the tips of the sea star arm (200 μg trypsin inhibited/mg total protein).

In conclusion, an inhibitor which inactivates trypsin but not chymotrypsin has been detected in the coelomic fluid of the sea star *Asterias forbesi.* The above data suggest that the trypsin inhibitor within the coelomic fluid of this invertebrate resembles the Kazal-type inhibitors of vertebrates. Although the physiological function of the protease inhibitor remains unclear, it may involve scavenger activity in neutralizing trypsin, which appears in the coelomic fluid, or host defense mechanisms.

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# A BLOOD-SUCKING SNAIL: THE COOPER'S NUTMEG, *CANCELLARIA COOPERI* GABB, PARASITIZES THE CALIFORNIA ELECTRIC RAY, *TORPEDO CALIFORNICA* AYRES

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## ABSTRACT

Feeding habits of the over 200 living species of Cancellariidae are largely unknown. The Cooper's nutmeg, *Cancellaria cooperi*, was seen by divers on the dorsal surface of Pacific electric rays, *Torpedo californica. C. cooperi* was observed to parasitize electric rays in the laboratory. The snails made small cuts on the ray's ventral surface and inserted their proboscises into the wounds. Snails were also observed to insert their proboscises into the mouth, gill slits, and anus, as well as previously existing wounds. After insertion of the proboscis, the snails appeared to suck blood from the ray. In Y-maze experiments, the snails actively sought out electric rays, but not other common California bottom fishes. Host location appeared to be by chemosensory means. In the absence of electric rays, snails remained buried in the sand without moving for at least 12 days. Field observations indicate that snails may travel as much as 24 meters in search of rays.

## INTRODUCTION

The family Cancellariidae comprises over 200 species of marine neogastropod mollusks inhabiting subtidal and bathyal sand and mud bottoms (Harasewych and Petit, 1982, 1984). Mechanisms of feeding in the Cancellariidae are almost entirely unknown (Harasewych and Petit, 1982, 1984; Petit and Harasewych, 1986). Early workers suggested that the snails feed on "soft-bodied microorganisms" (Graham, 1966; Olsson, 1970), but the snails' radular structure, relatively simple digestive system, long extensile proboscis, and accessory salivary glands are associated with a carnivorous diet (Harasewych and Petit, 1982, 1984). These and other features suggest that they are piercing fluid feeders (Harasewych and Petit, 1982; Petit and Harasewych, 1986). The snails also possess organs associated with distance chemoreception (Harasewych and Petit, 1982, 1984; Kohn, 1983). Previous reports of feeding are limited to single observations of cancellariids found on a bivalve mollusk, a gastropod, and pieces of fish and squid eggs in an aquarium (Cernohorsky, 1972; Talmadge, 1972; Garrard, 1975). Here we report that *Cancellaria cooperi* GABB, the Cooper's nutmeg, parasitizes the Pacific electric ray, *Torpedo californica* AYRES, by sucking the ray's blood. This is the first known report of gastropod mollusks parasitic upon fishes.

## MATERIALS AND METHODS

Field observations and collections were made during scuba dives at depths of 20–22 m at an artificial reef ("Torrey Pines #1" Lat *32°53′12″N* Long *117°50′50″W;* Cali-

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fornia Fish and Game Bulletin 45319-800 4-77-20m.OSP). Individuals of *C. cooperi* of 5–8 cm total shell length were collected by hand at a depth of 20.5 m. Snails were maintained in a 120-l aquarium equipped with running refrigerated seawater—about 14°C—with about 8 cm of sand on the bottom. Rays weighing 1.4–9.1 kg were collected with a hand net by the authors or with gill nets by local fisherman. They were maintained in a 1200-l aquarium equipped with running seawater until use in experiments.

For laboratory observations of behavior, snails were placed in a second 120-l aquarium, also provided with running seawater and 8 cm of sand. Rays were placed in this aquarium with the snails. In some cases, rays were first transferred to a 160-l aquarium and anesthetized by the addition of approximately 0.025 g/l of Tricane Methanesulfonate (Crescent Research Chemicals, Paradise Valley, Arizona).

Experiments were conducted in a plastic Y-maze with walls 5 cm high. The base of the 'Y' was 49.5 cm long and each arm was 25 cm long. Seawater from a tank containing an electric ray or other fish was siphoned into one arm, the "stimulus" arm, of the maze at a rate of 0.7 l/min.; seawater from a tank containing no animals was siphoned into the other, "control" arm at the same rate. The stimulus arm was chosen for each trial by a coin toss. The subject snail was placed in an experimental tray at the base of the 'Y' and allowed to acclimate for 3 min. Snails that did not emerge from their shells after 3 min were allowed to acclimate until they did so, up to a total acclimation period of no more than 5 min. By definition, a "response" occurred when the subject snail moved 5 cm into either arm within 30 min following the acclimation period. In positive responses, snails entered the stimulus arm; in negative responses, they entered the control arm. The maze was washed after each trial.

To test the alternate hypotheses that snails locate rays by chemoreception or via the rays' electric field, we performed Y-maze experiments as described above, except that in one treatment—the "ray water" treatment—seawater from a ray's aquarium was transferred by bucket to a reservoir, then siphoned into the stimulus arm of the maze; plain seawater was siphoned into the other arm. In the control treatment, plain seawater was siphoned into both arms of the maze.

Movements by snails in the maintenance aquarium were monitored by placing toothpicks upright in the sand directly adjacent to buried snails and checking the position of the snails relative to that of the toothpicks daily for 12 days. Movements during the intervening periods could be detected because the snails leave distinct trails in the sand.

## RESULTS

During scuba dives, we observed *Cancellaria cooperi* individuals resting on the dorsal surface of partially buried Pacific electric rays (*Torpedo californica*). As many as seven snails were seen on a single ray, and 16 of the 23 individuals of this rare snail which we collected were found in the presence of rays.

When maintained in an aquarium with an electric ray, *C. cooperi* individuals approached the ray and repeatedly touched its surface with extended tentacles. Within a few minutes, the proboscis was extended and used to probe the ray's surface. In most cases, snails made small cuts on the ray, presumably with the radula which is well suited for piercing (Fig. 1), and inserted the proboscis into the resulting wound. Sometimes the proboscis was inserted in the ray's mouth, gill slits, anus, or into open wounds, if already present. The proboscis was usually inserted on the ventral surface of the ray even if the ray was anesthetized and placed on its back. After being inserted, the proboscis appeared to pulsate, as if pumping fluid. This behavior was maintained

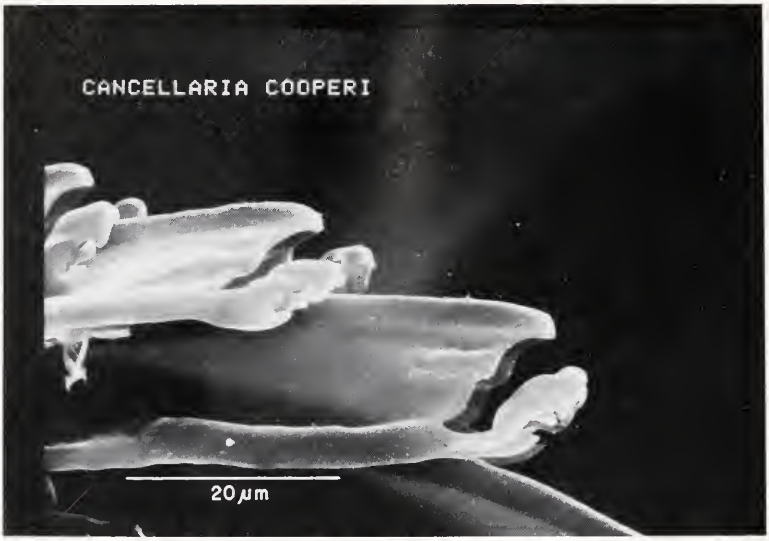

FIGURE 1. Lateral view of the central radular teeth of *Cancellaria cooperi*. Distal end of the radular ribbon is shown. No lateral teeth are present. Scale bar represents 20 μm. Scanning electron micrograph courtesy of M. G. Harasewych.

for periods of up to 40 minutes. On a few occasions, snails which were subsequently forced by rough handling to retract into their shells exuded a red fluid which appeared to be ray blood upon microscopic examination. Rays showed no apparent response to the snails' activities.

In Y-maze experiments, snails were attracted to water siphoned from tanks containing rays (Table I). Snails did not approach other benthic fishes common to the habitat (Table II), though recently we observed *C. cooperi* feeding on the angel shark, *Squatina californica*, in the laboratory.

Snails were attracted to ray water even if it was not siphoned directly from the ray's aquarium to the maze (Table III). Snails in the aquarium also moved to a microscope slide which had been scraped over the ray's surface to collect mucus, though

TABLE I

*Results of Y-maze experiments with* Cancellaria cooperi, *using the California electric ray,* Torpedo californica *as the stimulus*

| Snail no. | Response + | NR | − |
|---|---|---|---|
| 1 | 4 | 2 | 1 |
| 2 | 2 | 4 | 1 |
| 3 | 4 | 1 | 2 |
| 4 | 4 | 2 | 1 |
| 5 | 4 | 3 | 0 |
| Total | 18 | 12 | 5 |

There was no significant difference in behavior among snails (rows by columns *G*-test). Positive responses were significantly more frequent than negative responses (two-tailed binomial $P < 0.01$).

TABLE II

*Results of Y-maze experiments with various common California bottom fishes used as stimuli*

| Species | Response<br>+ | NR | – |
|---|---|---|---|
| *Torpedo californica* (electric ray) | 21 | 6 | 1 |
| *Urolophus halleri* (stingray) | 0 | 8 | 0 |
| *Platyrhinoidis halleri* (thornback ray) | 0 | 8 | 0 |
| *Eptatretus stoutii* (hagfish) | 0 | 8 | 0 |
| *Paralichthys californicus* (halibut) | 0 | 8 | 0 |

Differences in snail response among fish species are statistically significant (rows by columns $G = 49.8$, $P < 0.01$).

they did not respond when pieces of fresh ray flesh, with skin attached, were placed in the aquarium.

When maintained in aquaria in the absence of rays, snails remained buried in the sand without moving for the entire 12 days of observation, but snails moved at speeds of over 14 cm $\text{min}^{-1}$ when approaching rays in the maze. In the field, we have retraced the trails left by moving snails for distances of as much as 24 meters from rays.

## DISCUSSION

We conclude that *Cancellaria cooperi* is a specialized suctorial parasite of the Pacific electric ray, *Torpedo californica*, and perhaps of certain other bottom fishes such as *Squatina californica*. Though rare, the snails have been observed on electric rays by other divers (M. Tegner, Scripps Inst. Oceanography, pers. comm.; R. Kiwala, Monterey Bay Aquarium, pers. comm.). Location of rays is by chemosensory means, rather than by detection of the ray's electric field, since snails are attracted to ray water even if not directly connected by siphon to the ray tank. The chemical(s) sensed by the snails are apparently contained in the ray's surface mucus.

The snails probably remain buried in the sand for long periods until rays come

TABLE III

*Results of Y-maze experiments with* Cancellaria cooperi *in which seawater from a ray's tank was transferred by bucket to a reservoir, then siphoned into the stimulus arm of the maze; plain seawater was siphoned into the other arm. In control experiments, plain seawater was siphoned into both arms of the maze*

| Treatment | Response<br>+ | NR | – |
|---|---|---|---|
| Ray water | 7 | 1 | 0 |
| Control | 0 | 8 | 0 |

The differences in response between treatments are statistically significant (rows by columns $G = 15.9$, $P < 0.01$).

within chemosensory distance. By minimizing movement, snails presumably also minimize expenditure of energy in the absence of a food supply. The snails are capable of chemoreception over long distances, and can move rapidly to locate the ray once it is sensed. The ray, like the angel shark, is well suited as host for the snails since both fishes commonly remain on the bottom, partially buried, for long periods of time. The ray's apparent lack of response is in keeping with its behavior: Rays often will not respond even when prodded by divers. Furthermore, at least some cancellariids possess a hypobranchial gland which may be involved in toxin production (Harasewych and Petit, 1984). It is possible that the secretions of the hypobranchial gland of *C. cooperi* act as a local anesthetic and/or anticoagulant.

This appears to be the first published report of a gastropod mollusk parasitizing a fish (C. Hickman, University of California, Berkeley, pers. comm.; R. Robertson, Academy of Natural Sciences, Philadelphia, pers. comm.).

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